

CROSS-IDIOTYPIC SPECIFICITY OF RAT ANTIBODIES TO
STREPTOCOCCAL A CARBOHYDRATE

by

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A THESIS

Presented to the Department of Microbiology and Immunology
and the Graduate Division of the University of Oregon Health Sciences Center
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

July 1975

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ACKNOWLEDGMENTS

This thesis would not have been possible without the scientific insight and continued support from my advisor, Dr. Gerrie A. Leslie, and I thank him for both.

Dr. Marvin Rittenberg made significant contributions to the work involved in this thesis in terms of ideas and overall interest which I greatly appreciate.

I wish to thank the members of my Thesis Committee, Drs. Leslie, Rittenberg, Koler, Burger and Bardana for their suggestions and support of my work, and Ms. Rita Moran for final typing of this manuscript.

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INTRODUCTION

Statement of the Problem

Oudin first proposed the term "idiotypic specificity" to designate a unique individual antigenic determinant of immunoglobulins which was not present on antibodies of the same specificity from other individuals nor on antibodies with different specificity from the same individual. (Oudin, 1966). This original definition, however, provides no explanation for the increasing frequency of detectable cross-idiotypic specificity of antibodies and must be altered in consideration of such cross-specificity. In more general terms, therefore, idiotypic determinants may be defined as the expression of a portion of the variable region gene pool selected in an individual during the antibody response to a particular immunogen. The available variable region gene pool and the expression of this pool upon antigenic stimulation may obviously vary in different species of animals and between different individuals of the same species reflecting the genetic composition of the individual or species and the nature of the antigenic stimulus.

A number of antigen-antibody systems in a variety of experimental animals has been employed in the analysis of idiotypic determinants of antibody molecules. However, few, if any, offer the advantages of the streptococcal carbohydrate:anti-streptococcal carbohydrate system. Characteristics of rabbit anti-streptococcal carbohydrate antibodies

produced upon immunization and factors associated with the magnitude and degree of restriction of these antibodies have been reviewed by Krause (1970).

It was of interest, therefore, to develop a similar system in the rat by which genetic influences on both the magnitude and degree of restriction of the antibody response could be investigated. More important, however, the development of an effective technique for the isolation of anti-streptococcal A carbohydrate antibodies (anti-SACHO) of restricted heterogeneity would allow the procurement of anti-idiotypic antisera and the subsequent analysis of idiotypic determinants on rat anti-SACHO antibodies. In more specific terms, the objectives of this research were:

1. To define certain characteristics of the humoral response of rats to SACHO (e.g. anti-SACHO antibodies of restricted heterogeneity).
2. To confirm or deny genetic influences on both the magnitude and degree of restriction of rat anti-SACHO antibodies.
3. To develop an effective technique for the isolation of rat anti-SACHO antibodies of restricted heterogeneity.
4. To elicit anti-idiotypic antisera and analyze the expression of idiotypic determinants on rat anti-SACHO antibodies.
5. To examine the rat interstrain antibody response and idiotypy to SACHO.
6. To specifically purify rat anti-SACHO antibodies of restricted

heterogeneity to allow an amino acid sequence analysis of isolated heavy and light chains.

Review of the Literature

The development and subsequent analysis of a specific antigen-antibody system represents the inclusion of several specialized disciplines within the field of immunology. A review in detail of each contributing area, however, is not of necessity to the general understanding of the methodology employed in development of the system or conclusions drawn from its analysis. Accordingly, the references cited will be selective yet representative and may reflect the author's own unintentional bias.

A. The induction of anti-carbohydrate antibodies of restricted heterogeneity:

The structural heterogeneity of immunoglobulins and the molecular and functional heterogeneity of specific antibodies has long delayed analysis of antibody combining sites and structure-function relationships between antigens and antibodies. Only recently, experimental approaches have succeeded in generating antibodies with restricted heterogeneity in animals. Early observations on human antibodies to a variety of carbohydrate antigens demonstrated a structurally restricted antibody response. Individual antigenic specificity was demonstrated for anti-human blood group A, anti-human blood group B, anti-dextran, anti-levan and anti-teichoic

acid antibodies isolated from human sera (Kunkel, Mannik and Williams, 1963). It was anticipated then and subsequently confirmed that immunization of experimental animals with carbohydrate antigens would elicit anti-carbohydrate antibodies of restricted heterogeneity. Undoubtedly, the most extensively characterized humoral response of restricted heterogeneity has been induced by streptococcal carbohydrates. Initial observations with rabbit anti-streptococcal carbohydrate antibodies demonstrated an electrophoretic uniformity and a serum concentration of the anti-carbohydrate antibodies not unlike that of myeloma proteins (Osterland, Miller, Karakawa and Krause, 1966). More recent studies have confirmed the induction by streptococcal vaccines of anti-carbohydrate antibodies of restricted heterogeneity in sharks (Clem and Leslie, 1971), chickens (Leslie and Clem, 1973), Japanese quail (Leslie, unpublished observations), mice (Braun, Kindred and Jacobson, 1972) and rats (Stankus and Leslie, 1974a).

Factors influencing the occurrence of anti-carbohydrate antibodies of restricted heterogeneity:

The mechanism(s) of induction of structurally restricted anti-carbohydrate antibodies after immunization with bacterial vaccines remains unexplained. Proposed explanations by Haber (1971) include: 1) the genetic deletion of the capacity to make more than a limited number of antibodies, 2) overgrowth of available space by a single

clone capable of more efficient growth, 3) emergence of a clone with receptors having a high affinity for antigen, and 4) an interplay between tolerance and immunity.

Undoubtedly, antibody heterogeneity is in part a reflection of antigen heterogeneity (Haber, Richards, Spragg, Austen, Vallotton and Page, 1967). Although streptococcal carbohydrates are complex polysaccharides, fewer antigenic sites are available than on most proteins. This restriction in antigenic heterogeneity may restrict the diversity of antibody production. The studies of Pappenheimer, Reed and Brown (1967) are consistent with this view.

Krause (1970), in reviewing the induction of anti-streptococcal carbohydrate antibodies of restricted heterogeneity, mentioned four variable factors which may influence the occurrence of monodisperse antibodies. These are: 1) the route of immunization, 2) the physical state of the antigen, 3) prior sensitization and 4) the genetic makeup of the animal.

Although a detailed analysis of the advantages and disadvantages of alternate routes of immunization with bacterial vaccines has never been published, subjective impressions by Lancefield (Krause, 1970) were that alternative routes and the use of adjuvants were not as effective as intravenous injection of a heat-killed streptococcal vaccine. Krause (1970) in examining the most effective composition of a bacterial vaccine for the induction of anti-streptococcal carbohydrate antibodies,

noted that a pepsin-treated vaccine of whole streptococci with the carbohydrate as the outermost cell wall component was able to stimulate a greater immune response in rabbits than isolated cell walls. Additionally, the purified soluble carbohydrate was found to be non-antigenic in rabbits, conceivably representing a toleragenic stimulus. A schematic representation of this pepsin-treated vaccine of group A streptococci and the live streptococci from which the vaccine is prepared is presented in Fig. 1.

Genetic factors are undoubtedly one of the most important influences regulating the magnitude and degree of restriction of the response of animals to streptococcal vaccines. Selective breeding of high- and low-response breeding pairs demonstrated genetic influences on the magnitude and degree of restriction of both rabbit antibodies to streptococcal carbohydrates (Braun, Eichmann and Krause, 1969; Braun, Kjems and Cramer, 1973; Eichmann, Braun and Krause, 1971) and rat antibodies to SACHO (Stankus and Leslie, 1974a, 1975). Furthermore, appropriate breeding studies revealed independent genetic influences on the magnitude of the response of rabbits to streptococcal carbohydrates in comparison to the degree of restriction of rabbit anti-carbohydrate antibodies (Eichmann, Braun and Krause, 1971).

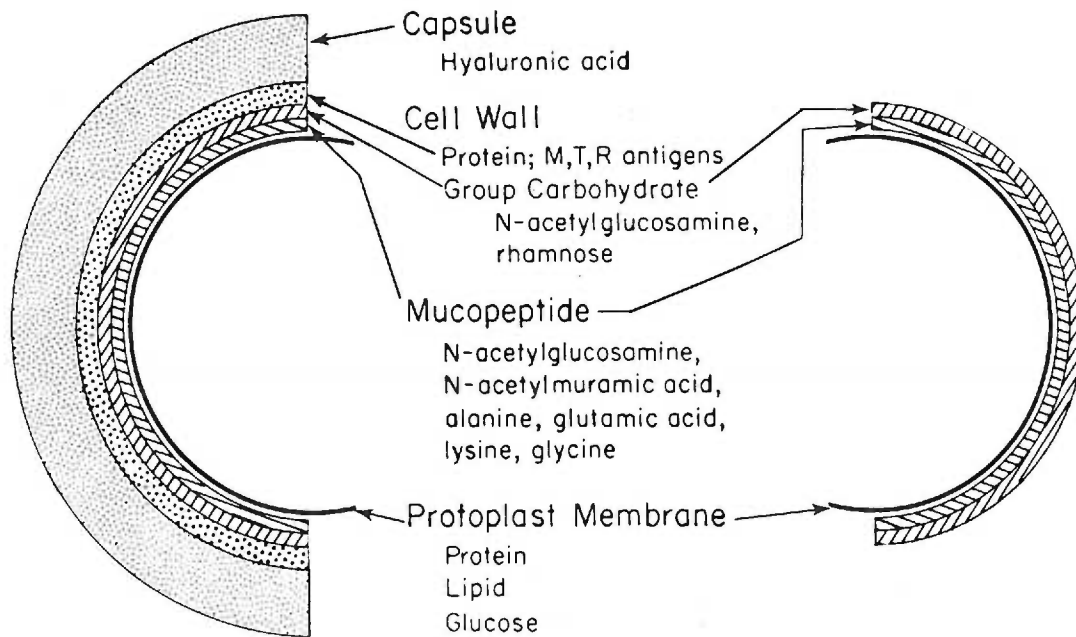
In view of numerous reports of histocompatibility-associated or histocompatibility-linked immune response genes, reviewed by McDevitt and Benacerraf (1969) and more recently by Gasser and Silvers (1974),

Figure 1

Schematic representation of live group A streptococci and heat-killed
pepsin-treated streptococcal vaccine.

Living Group A Strep.

Dead Vaccine



it is conceivable that immune response genes to streptococcal carbohydrates are also associated with or linked to histocompatibility genes. However, an analysis of the response to SACHO of several inbred mouse strains demonstrated the non-association of the histocompatibility locus with the magnitude of the immune response to SACHO (Braun, Kindred and Jacobson, 1972). Two inbred mouse strains of the same histocompatibility haplotype demonstrated both a high and low anti-SACHO response.

B. THE ISOLATION FROM SERUM OF ANTI-CARBOHYDRATE ANTIBODIES OF RESTRICTED HETEROGENEITY:

The isolation from serum of antibodies demonstrating molecular uniformity has widely been achieved using specific immunoadsorbents and subsequent gradient elution employing a number of eluting reagents. Parker and Briles (1970) and Eichmann and Greenblatt (1970) fractionated restricted antibodies from streptococcal antisera by means of affinity chromatography. McCarty (1958) has shown that the terminal β -N-acetyl-glucosaminide residues are the immunodominant determinants of SACHO. Therefore, an immunoadsorbent was prepared by coupling p-aminophenyl- β -N-acetyl-glucosaminide to Sepharose activated with cyanogen bromide as originally described by Axen, Porath and Ernback, 1967 and Porath, Axen and Ernback, 1967. The specifically adsorbed antibodies were eluted with a gradient of N-acetylglucosamine at neutral pH. More recently, Eichmann and Greenblatt (1971) employing an acetic acid/

sodium chloride gradient were able to fractionate rabbit antibodies of restricted heterogeneity to streptococcal carbohydrates. They concluded that the net electrical charge of an antibody bears a reciprocal relationship to its relative binding affinity for the immunoabsorbent column. In general, the greater the electrophoretic mobility of an antibody component, the lower the relative affinity for the immunoabsorbent column.

C. IDIOTYPIC DETERMINANTS OF IMMUNOGLOBULINS:

The ability to elicit antibodies with molecular uniformity to carbohydrate antigens and the subsequent isolation of these antibodies from antisera, allows the production of an anti-idiotypic antiserum and an analysis of idiotypic determinants on antibody molecules. Oudin (1966) first defined idiotypic determinants as antigenic determinants on immunoglobulins which were not present on antibodies to the same antigen from other individuals, nor on antibodies to different antigens from the same individual.

Conforming to this original definition, idiotypic determinants have been established in myeloma proteins (Slater, Ward and Kunkel, 1955; Korngold and Lipari, 1956), macroglobulins (Korngold and Van Leeuwen, 1957a, b; Boerma and Mandema, 1957), and Bence-Jones proteins (Stein, Nachman and Engle, 1963) as well as in human (Kunkel, Mannik and Williams, 1963) and rabbit (Gell and Kelus, 1964; Kelus and Gell, 1968) antibodies to a variety of antigens.

Cross-idiotypic specificity of antibodies:

Although initial experiments on idiotypic determinants of rabbit antibodies to streptococcal carbohydrates confirmed unique individual antigenic specificity of rabbit anti-carbohydrate antibodies (Braun and Krause, 1968), later observations demonstrated cross-idiotypic specificity for certain homogeneous streptococcal carbohydrate antibodies (Eichmann and Kindt, 1971; Braun and Kelus, 1973). This cross-specificity was only found within single families of immunized rabbits and anti-carbohydrate antibodies from outbred rabbits lacked this shared idio type. Therefore, although rabbit anti-streptococcal carbohydrate antibodies might share common idiotypic determinants, this cross-specificity is detectable only in genetically related animals reflecting the importance of a similar genotype in the expression of a similar idio type. This observation by itself represents but a minor discrepancy with the original definition of an idio type.

The detection of idiotypic cross-reactions on rabbit anti-benzoate antibodies (Hopper, MacDonald and Nisonoff, 1970), rabbit anti-Salmonella abortus equi antibodies (Oudin and Bordenave, 1971), mouse anti-streptococcal carbohydrate antibodies (Briles and Krause, 1974), rat anti-SACHO antibodies (Stankus and Leslie, 1974b), human IgM cold agglutinins (Williams, Kunkel and Capra, 1968; Williams, 1971) and human monoclonal anti- γ -globulins (Franklin and Frangione, 1971; Kunkel, Agnello, Joslin, Winchester and Capra, 1973) however, demonstrates the occurrence

of cross-reactive idiotypes among antibodies from both related and unrelated individuals and expands the scope of cross-idiotypic specificity beyond the confines of genetic relatedness.

It is apparent from the above observations that the original definition of an idiotypic encompassing individual specificity must now be revised. A single feature of idiotypes, which has served as a basis for its distinction from allotypes, remains unaltered by published observations. The inability to detect an idiotypic cross-reaction between antibodies from the same individual to different non-cross-reacting antigens, supports the distinction of idiotypic from allotype or isotype by demonstrating the importance of the antibody combining site in conferring an idiotypic specificity.

The unrelatedness of idiotypic determinants on antibodies of different specificity from the same individual is well documented by the work of Kunkel, Mannik and Williams (1963) who observed that anti-blood group A substance and anti-levan, purified from the serum of a single human, showed unrelated idiotypic determinants. Similarly, Oudin and Michel (1963) found that idiotypic determinants on rabbit anti-Salmonella antibody failed to cross-react with those on anti-pneumococcal antibody from the same rabbit. In a subsequent experiment, antibodies from a single donor, specific for Salmonella typhi and Salmonella tranoroa, respectively, were found to have unrelated idiotypic specificities (Oudin and Michel, 1969). The available data, therefore, provides no

evidence for idiotypic cross-reactions among antibodies of different specificity from the same individual.

Two related questions concerning idiotypic cross-specificity which have only recently been answered are: 1) can physicochemically distinct antibodies to the same antigen from the same donor share common idiotypic determinants and 2) can antibodies to different antigens from different individuals show idiotypic identity? In answer to these questions, Thunberg and Kindt (1974) demonstrated an idiotypic cross-reaction between two distinct streptococcal carbohydrate antibodies from an individual rabbit. In like manner, the observation of shared idiotypic determinants on antibodies from different rabbits to Salmonella abortus equi and tobacco mosaic virus (Bordenave, 1973a, b) extend yet further the realm of cross-idiotypic specificity. It should be stressed, however, that the occurrence of this interantigenic idiotypic cross-specificity, after examination of as many as 1260 antisera was less than 1%.

Localization of idiotypic determinants on the antibody molecule:

Any attempt to incorporate the aforementioned observations on idiotypic determinants and cross-idiotypic specificity within the existing theories of the generation of antibody diversity, must consider in each instance the localization on the antibody molecule of the idiotypic determinants described. One would expect the majority if not

the entire idiotypic determinant to be associated with variable sequences on the antibody. Indeed, in view of the nature of idiotypes, the association of the determinants with the combining site of the antibody molecule would be expected.

Quantitative analysis of the rabbit anti-p-azobenzoate:anti-idiotypic antibody system indicate that the combining site of the anti-benzoate antibody is part of or close to an idiotypic determinant. The ability of benzoate derivatives to significantly inhibit the reaction of ^{125}I -labeled $\text{F}(\text{ab}')_2$ fragments of anti-benzoate antibody with its anti-idiotypic antiserum and the non-inhibitory nature of unrelated compounds clearly suggests the antibody combining site as contributing to the idiotypic determinant (Brient and Nisonoff, 1970). Similarly, the antibody-combining site of human cold agglutinins demonstrating cross-idiotypic specificity has been implicated in determining their idiotypic specificity. The idiotypic:anti-idiotypic reactions were blocked when the cold agglutinins were bound to the I antigen on the red blood cell (Williams, Kunkel and Capra, 1968). Indeed, the reaction of mouse antibodies to DNP (Sirisinha and Eisen, 1971), phosphorylcholine (Sher and Cohn, 1972), dextran (Carson and Weigert, 1973) and SACHO (Briles and Krause, 1974) with their respective anti-idiotypic antisera, have been inhibited by antibody-specific hapten.

Observations by Kelus and Gell (1968), however, suggest that the antibody combining site in some instances may not be an important

idiotypic determinant. Rabbit anti-Proteus vulgaris antibodies still reacted with anti-idiotypic antisera when the antibodies were combined with Proteus. However, since their assay employed bivalent anti-Proteus antibody, reaction of the anti-idiotypic antibody with antigenically unbound antibody combining sites may explain the lack of inhibition.

In addition to the association of idiotypic determinants with the combining site of antibody molecules, clear evidence for chain localization of idiotypes has been reported. Antisera made to light chains or Bence-Jones proteins readily produced anti-idiotypic antibodies (Mannik and Kunkel, 1962). This was particularly true of the lambda-type light chains. For example, Grey, Mannik and Kunkel (1965) examined the heavy and light chains from eleven human IgG myeloma proteins for their reactivity with anti-idiotypic antibody directed to each intact protein. In three of five myelomas with lambda light chains, the idiotypic specificity was localized only to the light chains. Lines of identity by the Ouchterlony method were observed between the isolated lambda light chains and the intact protein in all three cases, suggesting the idiotypic specificity was entirely due to determinants on the light chains. The two remaining myelomas with lambda light chains expressed their idiotypic determinants on the heavy chains only.

In contrast to lambda light chains, analysis of isolated kappa chains from six myelomas demonstrated no reactivity with anti-idiotypic antisera directed to the intact myeloma protein. In two cases, the

isolated heavy chains formed a line of partial identity by Ouchterlony with the myeloma protein. In summary, therefore, while isolated lambda light chains will often react with anti-idiotypic antisera directed to the intact immunoglobulin, kappa light chains generally do not.

Genetic analysis of the expression of idiotypic determinants:

Genetic evaluation of the expression of idiotypic determinants associated with the immune response of mice to SACHO has demonstrated the Mendelian segregation of a mouse antibody idotype (Eichmann and Berek, 1973). This idotype was expressed in over 90% of all A/J mice immunized with group A streptococcal vaccine but was lacking in BALB/cJ mice. The expression of this idotype was found to be controlled by a gene in the heavy chain linkage group and segregation of the idotype in backcross experiments followed the segregation of the heavy chain allotype.

Similarly, Nisonoff (1974) has reported the cross-specificity of idiotypic determinants on mouse antibodies to p-azophenylarsonate. Shared idiotypic specificity was demonstrated for inbred mouse strains A/J, A/He and AL/N. In contrast, BALB/c, SJL/J, C57BL/cdj and NZB mice lacked this cross-reactive idotype. The gene controlling this idotype was closely linked to the IgC_H locus suggesting a close linkage of V_H and C_H genes.

Sher and Cohn (1972) surveyed the anti-phosphorylcholine responses

of different inbred mouse strains and reported the expression of a cross-reactive idio-
type in several strains (BALB/c, NZB, SJL) and its lack of
expression in other strains (A/J and CE/J). The inheritance of this idio-
type was shown to be determined by a gene linked to the chromosome carry-
ing the mouse heavy chain allotype locus. Backcross data suggested the
additional participation of a second, independently segregating gene in
the determination of this idio-
type.

The passive suppression of idiotypic determinants:

In several antigen-antibody systems, the specific suppression of a
cross-reactive idio-
type with anti-idiotypic antisera has produced vary-
ing results in relation to the subsequent antibody response. For example,
Hart, Pawlak and Nisonoff (1973) demonstrated that different individual
suppressed A/J mice produced anti-p-azophenylarsonate antibodies differ-
ing in idio-
type. Although the cross-reacting idio-
type was suppressed
almost completely, the titer of anti-hapten antibody was comparable to
that of control A/J mice which received non-specific IgG in place of the
anti-idiotypic IgG. Fab' and F(ab')₂ fragments of anti-idiotypic IgG
were ineffective in causing suppression (Pawlak, Hart and Nisonoff, 1973).
This was attributed to either a rapid clearance of the fragments from the
serum or to a requirement for activation of complement in causing suppres-
sion. Interestingly, this observation is in contrast to immunoglobulin
class suppression studies in chickens, whereby F(ab')₂ anti-μ antibodies

can mediate suppression (Leslie and Martin, 1973), suggesting that antibody-complement cytotoxicity is not involved.

Eichmann (1974), in examining the anti-SACHO response of A/J mice, was able to suppress the production of a cross-specific idiotypic, A5A, by anti-idiotypic antibody directed against antibody A5A. Mice pretreated with anti-A5A showed only slightly reduced concentrations of antibodies to SACHO but were strongly suppressed for the A5A idiotypic. Pretreatment of mice with an anti-idiotypic antibody against a heterologous idiotypic did not affect the production of idiotypic A5A. The suppressive effect of anti-idiotypic antibody was mediated exclusively by guinea pig IgG₂ antibodies which have been shown to fix complement (Bloch, Kourilsky, Ovary and Benacerraf, 1963) and possess a specific receptor for macrophages (Berken and Benacerraf, 1966). This observation suggested that precursor cells to which anti-idiotypic antibodies are bound, are either lysed by complement, phagocytized by macrophages, or both. Blocking of receptors by anti-idiotypic antibody was discounted as an explanation of the results since guinea pig IgG₁, anti-idiotypic antibodies were unable to cause suppression. These results then are similar to those of Hart, Pawlak and Nisonoff (1973). As the anti-arsonate antibodies of suppressed A/J mice had individually specific idiotypes, the anti-SACHO antibodies of suppressed A/J mice had individually specific isoelectric spectra.

In contrast to the above observations, Cosenza and Kohler (1972) observed that passive administration of anti-idiotypic antisera was effective in suppressing completely the response of Balb/c mice to phosphorylcholine. Moreover, this anti-idiotypic antiserum, directed to Balb/c mouse myeloma protein TEPC-15 with binding activity for phosphorylcholine, did not suppress the antibody response to sheep erythrocytes. Suppression resulted from inhibition of induction rather than inhibition of plaque-formation since anti-idiotypic antiserum added to immune spleen cells one or more days after immunization had no suppressive effect. The authors concluded that inhibition of response to phosphorylcholine was probably due to anti-idiotypic antibodies binding to receptor molecules thereby preventing stimulation of cell clones by antigen. The complete inhibition of the anti-phosphorylcholine response by anti-idiotypic antibody suggested the occurrence in each mouse of cell clones bearing receptors of restricted specificity for antigen.

Amino acid sequence analysis of idiotypically-related antibodies:

Undoubtedly the most stringent proof for homogeneity of specific antibodies is obtained from amino acid sequence data. For example, rabbit anti-DNP antibodies have been shown to possess a sequence heterogeneity indistinguishable from normal IgG (Doolittle, 1965). In contrast, however, the sequence variability of rabbit anti-streptococcal

and anti-pneumococcal carbohydrate antibodies is not unlike the molecular restriction of myeloma proteins (Fleischman, 1971; Hood, Waterfield, Morris and Todd, 1971; Jaton, Waterfield, Margolies and Haber, 1970, 1971). The molecular restriction of anti-carbohydrate antibodies, therefore, provides a means to analyze sequence homologies between antibodies with similar idiotypic specificity. In reference to this analysis, myeloma proteins with anti-IgG activity belonging to idiotypically similar groups were shown to have heavy chains of the same V_H subgroup (Kunkel, Agnello, Joslin, Winchester and Capra, 1973). These studies have recently been extended to show that a relationship also exists between cross-idiotypic specificity and V_L subgroup (Kunkel, Winchester, Joslin and Capra, 1974). Thunberg and Kindt (1974) in analyzing the N-terminal amino acid sequence of two idiotypically indistinguishable light chains isolated from distinct antibodies to streptococcal C carbohydrate from the same antiserum, observed only a single amino acid difference for the first 23 residues. Finally, Capra, Tung and Nisonoff (1975) have demonstrated that mouse anti-azophenylarsonate antibodies bearing a cross-reactive idio- type, possess identical amino acid substitutions in the first heavy chain hypervariable region (N-terminal amino acids 31-35), while anti-azophenylarsonate antibodies depleted of this cross-reactive idio- type did not possess this sequence. It was concluded, therefore, that the first heavy chain hypervariable region of the idiotypically-related azophenylarsonate antibodies

represented a selection on the basis of the cross-reacting idio-
type.

In summary then, the development of a carbohydrate:anti-carbohydrate system can allow not only an analysis of influences regulating the overall anti-carbohydrate response (e.g. genetic factors), but more important, both an analysis of idiotypic determinants associated with the humoral response and sequence analysis of specific anti-carbohydrate antibodies. Extensive idiotypic studies in association with amino acid sequence analysis should provide insight into mechanisms regulating both the immune response in general and more specifically, the variability in response to carbohydrate antigens between individuals of the same strain, between strains of the same species and ultimately between different species. Observations of this nature will undoubtedly help to elucidate the mechanism(s) for the generation of antibody diversity.

MATERIALS AND METHODS

BACTERIAL STRAINS

1. Group A Streptococcus, strain J17A4. A lyophilized stock culture of group A Streptococcus, strain J17A4, was originally obtained from Dr. Richard Krause of the Rockefeller University.

MEDIA

1. Todd-Hewitt broth. This broth was prepared by dissolving 30 g dry powder (Difco) per liter of distilled water and sterilized in the autoclave for 15 min at 15 pounds pressure (121 C).

BUFFERS

1. Phosphate buffered saline (PBS). This buffer was prepared by dissolving 1.14 g Na_2HPO_4 , 0.27 g KH_2PO_4 and 8.5 g NaCl in 1 liter of distilled water. The final pH was 7.2.
2. Tris-buffered saline (TBS). This buffer was prepared by dissolving 1.32 g Trizma-HCl [Tris(hydroxymethyl)aminomethane Hydrochloride], 0.19 g Trizma-base, 1 g NaN_3 , 0.37 g EDTA and 8.3 g NaCl in 1 liter of distilled water. The final pH was 7.4. This buffer is commonly referred to in our laboratory as Sephadex buffer.

3. Immunodiffusion buffer. This buffer was prepared by dissolving 6.98 g sodium barbital, 6 g NaCl and 10 ml of a 1% thimersol solution in 1 liter of distilled water. The pH was adjusted to 7.4 with HCl and the ionic strength was 0.15.
4. Immuno-electrophoresis buffer. This buffer was prepared by adding 5.4 g sodium barbital, 2.3 g $\text{NaC}_2\text{H}_3\text{O}_2$, 58.2 ml of 0.1 N HCl and 10 ml of a 1% thimersol solution to 1 liter of distilled water. The final pH was 8.6 and the ionicity 0.05.

Group A streptococcal vaccine (GASV). Vaccine was prepared from lyophilized cultures of group A Streptococcus, strain J17A4, according to the method of Osterland, Miller, Karakawa and Krause (1966). Ten liters of an 18 hr Todd-Hewitt broth culture of the streptococci were heat-killed at 60 C for 90 min. The cultures were streaked on blood agar plates before heating to establish their purity and after heating to confirm that all streptococci were killed. The streptococci were collected by centrifugation (2500 g for 10 min) and the cells washed several times with sterile saline. The streptococcal pellet (8-10 g wet weight) was suspended in 250 ml of sterile saline, adjusted to pH 2 and containing 500 mg of pepsin. The mixture was incubated with periodic mixing for 2 hr at 37 C, centrifuged, and washed several times with sterile saline. The final product was suspended in sterile saline at a concentration of 10 mg per ml (dry weight) and cultured to

check for sterility (schematic representation, Fig. 1, p. 7).

Purified streptococcal A carbohydrate (SACHO). Carbohydrate was isolated from cell walls by hot formamide digestion. This procedure is given in complete detail elsewhere (Krause and McCarty, 1961). The carbohydrate was further purified from ultraviolet absorbing material by sodium sulfate precipitation and filtration through an analytical grade mixed bed resin. (Bio-Rad AG501-X8(D), 20-50 mesh). The purified carbohydrate had an absorbance of 0.05 at 280 nm in a concentration of 1 mg per ml.

Group A-variant streptococcal carbohydrate. Group A-variant streptococcal carbohydrate was the kind gift of Dr. L. W. Clem, University of Florida Medical School.

Experimental animals. Male and female, 250-350 g outbred Sprague-Dawley rats were originally supplied by Dr. K. F. Burns of the Tulane University Medical School. Inbred rat strains, August 28807 (Aug), Wistar Furth (W/Fu) and Marshall 520 (M520) were obtained from Charles River Breeding Laboratories. Copenhagen (Cop), (ARS/Sprague-Dawley); Fisher 344 (F344), (Simonsen Laboratories); Buffalo/Cr (Buf), (Tulane Medical School) and Brown Norway (BN), (Microbiological Associates) inbred strains were also obtained for this study. All

inbred rat strains were contracted through Sam Poiley, National Institutes of Health. The animals were maintained on Purina Rat Chow and allowed continual access to water.

Chicken, rabbit and goat anti-SACHO antisera. These antisera were the kind gift of Dr. G. A. Leslie, University of Oregon Medical School.

Specifically purified nurse shark anti-SACHO antibodies. These antibody preparations were the kind gift of Dr. L. W. Clem, University of Florida Medical School.

Immunization with GASV. For primary immunization, beginning at 60 days of age, the rats received three weekly intravenous injections of 1 mg of GASV, on consecutive days, for three weeks. After a 2-month rest period, secondary immunization by three intravenous injections of 0.2 mg of GASV, on consecutive days, was administered weekly for 3 weeks. Blood collections began 5 days after the last injection of each week.

Immunization with poly-glutamic acid-alanine (poly-GA). Primary immunization of rats with poly-GA (Pilot), beginning at 60 days of age, consisted of injections of an emulsified mixture of equal amounts of poly-GA dissolved in TBS and Freund's incomplete adjuvant supplemented with 4 mg per ml of killed Mycobacterium tuberculosis (Ra37)

(Difco). Each rat received 500 ug of poly-GA in a total volume of 0.4 ml distributed equally in all four footpads. Four weeks after priming, the rats were injected intradermally into the flank skin with 50 ug of poly-GA dissolved in 0.1 ml TBS. The intradermal injection was repeated one week after the first intradermal injection, into the opposite flank. The rats were bled four weeks after primary immunization (primary response antisera) and one week after the second intradermal injection (secondary response antisera) (Armerding, Katz and Benacerraf, 1974).

Selective breeding of high- and low-responding rats. From an initial population of 30 outbred Sprague-Dawley rats immunized with GASV, high- and low-responding rats were selected as parents and mated. High-responding rats chosen as parents generally represented animals with the highest precipitin response among littermates, while low-responding parents were those that produced <1 mg of precipitating antibody per ml of serum, after both primary and secondary immunization. These criteria were used in choosing parents for all brother-sister inbreedings.

Quantitative precipitin analysis of anti-SACHO. Quantitative precipitin tests were performed in TBS with 0.011 M EDTA by using 0.1 ml of anti-serum and a range of SACHO concentrations from 10 to 90 ug in a total volume of 1 ml. The precipitin tubes were incubated for 1 hr at 37 C

and stored at 4 C overnight. The washed precipitate was then dissolved in 2 ml of 0.1 N NaOH, and the optical density determined at 280 nm in an ACTA CIII spectrophotometer. Total protein in the precipitates was determined by using $E^{1\%}_{280\text{ nm}}$; 1 cm equals 14.6 for rat immunoglobulin (Binaghi and Benacerraf, 1964).

Detection and quantitation of rat anti-SACHO and anti-A-variant carbohydrate by reverse radial immunodiffusion (RRID). RRID plates were prepared by mixing either 1 or 2 ug of SACHO or A-variant carbohydrate with a 1% (w/v) solution of Seakem agarose (MCI Biomedical) in immunodiffusion buffer at 50 C. Three ml of this mixture was applied to a standard microscope slide; 6 ml, to a 7.5 x 5 cm slide, and 15 ml, to a 10 cm x 8 cm lantern slide. Wells 2.3 mm in diameter were cut in the solidified agarose just prior to use. Antisera were added to the wells and the reactions were allowed to proceed for approximately 24 hr at 4 C in a moist chamber. Dilutions of antisera whose antibody content was determined by a quantitative precipitin test were included on each plate. After incubation the diameters of the precipitin rings were measured to the nearest 0.1 mm with a Bausch and Lomb measuring magnifier and darkfield lighting (Leslie and Hattier, 1974).

Detection and quantitation of rat anti-poly-GA. Precipitating rat antibodies to poly-GA were detected and quantitated employing RRID in

agarose gel. With the exception of incorporating 1 ug/ml of poly-GA into the agarose in place of SACHO or A-variant carbohydrate, the procedure was performed exactly as described for the detection of rat anti-SACHO or anti-A-variant carbohydrate antibodies. Dilutions of antisera whose antibody content was determined by a quantitative precipitin test, were included as standards on each plate.

Double diffusion in agarose gel (Ouchterlony). Ouchterlony analysis was carried out on glass slides coated with 1% (w/v) Seakem agarose in immunodiffusion buffer and containing 2% (w/v) polyethylene glycol 4000 (Baker) to visibly enhance precipitation (Lundkvist and Ceska, 1972).

ELECTROPHORETIC PROCEDURES

1. Immuno-electrophoresis (IE). Immuno-electrophoretic analysis was performed in IE agar prepared by dissolving 10 g of Seakem in 1 liter of IE buffer, pH 8.6. The electrophoretic runs were made in a Gelman electrophoresis chamber at a constant current of 13 m amp per tray (27 x 5.5 cm) for 75 min.
2. Cellulose acetate electrophoresis (CA). Cellulose acetate electrophoresis was performed with a Beckman Microzone electrophoresis cell. Serum samples were supported in a cellulose acetate medium,

and electrophoretic runs were made at 250 V for 20 min in barbital buffer (Beckman, Buffer B-2), pH 8.6 and ionic strength 0.075. The cellulose acetate strips were stained for 10 min with Ponceau S, rinsed in 5% (v/v) acetic acid, dehydrated in absolute methanol, and cleared in an 18% (v/v) acetic acid/methanol solution.

3. Polyacrylamide gel disc electrophoresis. Disc electrophoresis was performed in alkaline urea-containing polyacrylamide gels prepared according to the method of Reisfeld and Small (1966). The gel dimensions were 7.5 x 0.7 cm. Protein samples, 100-400 ug, were dissolved in 100 ul of 10 M urea and layered onto the upper 2% acrylamide gel. The protein samples were electrophoresed at 1 m amp per tube until the tracking dye, bromophenol blue, reached the lower 4% acrylamide gel, at which time the current was increased to 2.5 m amp per tube. After the dye front had migrated to the bottom of the gels, the electrophoresis was allowed to continue for an additional 20 min. When the electrophoretic run was completed, the gels were fixed in 12.5% trichloroacetic acid for 30 min, stained for 30 min with a freshly prepared 1:20 dilution of 1% Coomassie blue in 12.5% trichloroacetic acid, and destained in 12.5% trichloroacetic acid. The urea solutions employed in preparing the gels and suspension of protein samples were filtered before use through the analytical grade, mixed-bed resin described previously for the purification of SACHO.

Preparation of normal rat IgG. Rat IgG was isolated from pooled whole rat serum by diethylaminoethyl-cellulose ion exchange chromatography (DEAE). The column was equilibrated with 0.005 M phosphate buffer (pH 7.6) and IgG was eluted from the anion exchange column with this same buffer and 0.015 M NaCl in phosphate buffer, pH 7.5, 0.01 M.

PREPARATION OF ANTISERA

1. Bovine anti-rat IgG. This antiserum was the kind gift of Dr. H. G. Carwile, Tulane University Medical School. The antiserum was prepared by suspending 5 mg of pooled normal rat IgG in complete Freund's adjuvant and injecting the emulsion intramuscularly into a 2-year-old Black Angus cow. The cow received one booster immunization of rat IgG in incomplete Freund's adjuvant 3 weeks after the initial injection, and blood was collected 3 weeks after the second injection. This antiserum had anti-heavy and anti-light chain specificities as judged by IE and Ouchterlony analysis.
2. Rabbit anti-whole rat serum (anti-WRS). This antiserum, the kind gift of Dr. H. G. Carwile, Tulane University Medical School, had antibody activity for rat IgM, IgG and IgA as judged by IE.
3. Rabbit anti-rat light chain antiserum. This antiserum was prepared by immunization of rabbits with rat light chains isolated from pooled normal rat IgG as described below. Each rabbit was injected

into the hind footpads with 1 mg of rat light chains mixed with an equal volume of complete Freund's adjuvant. Seventeen days later the rabbits were injected subcutaneously with 500 ug of rat light chains mixed with an equal volume of incomplete Freund's adjuvant. The antiserum obtained from weekly bleedings had anti-light chain activity only as determined by Ouchterlony analysis.

4. Chicken anti-human lambda light chain antiserum (anti- λ). This antiserum was prepared by immunization of chickens with λ Bence-Jones protein. Each chicken was injected subcutaneously in the neck region with 3 mg of λ light chains mixed with an equal volume of complete Freund's adjuvant. This methodology was continued at monthly intervals for 2 months and the chickens bled beginning 10 days after the third injection. The resultant antiserum had anti- λ activity and weak anti-kappa activity, and was therefore absorbed with a human IgG myeloma protein of the kappa type. After absorption the antiserum had only anti- λ activity as judged by Ouchterlony analysis. Examination of the absorbed anti- λ antiserum reacted against goat, cow, sheep, horse, and rat normal sera demonstrated single precipitin lines of partial identity to the reaction of the chicken antiserum and the λ Bence-Jones protein used for immunization. Precipitin lines of identity were observed when the absorbed chicken antiserum and a commercial anti-human λ

antiserum were reacted against the Bence-Jones protein used in preparation of the chicken antiserum (Orlans and Feinstein, 1971).

5. Rabbit anti-rat IgG antiserum. This antiserum was prepared in rabbits to normal rat IgG isolated by DEAE chromatography. Each rabbit was injected in the hind footpads with 3 mg of normal rat IgG mixed with an equal volume of complete Freund's adjuvant. One month later the rabbits were boosted subcutaneously with 2 mg of normal rat IgG mixed with an equal volume of incomplete Freund's adjuvant. Antisera obtained from weekly bleedings demonstrated anti-heavy and anti-light chain reactivity as judged by IE and Ouchterlony analysis.
6. Guinea pig anti-idiotypic antisera. These antisera were prepared in guinea pigs to specifically purified rat IgG anti-SACHO antibodies (R42G and R43G). Each guinea pig was given an intracardiac injection of 5 mg pooled normal rat IgG. Immediately after, 100 ug of anti-SACHO, mixed with an equal volume of complete Freund's adjuvant, were injected into the hind footpads of each animal. Twenty-five days later the guinea pigs were bled and these antisera absorbed to IgG antigen excess with pooled normal rat serum representing approximately 75 animals. After absorption, the antisera no longer reacted by Ouchterlony with pooled normal rat

serum, pooled normal rat IgG, or normal rat IgG heavy and light chains examined at concentrations ranging from 0.1 mg per ml to 10 mg per ml. These antisera, however, retained activity with their specific immunogens. Each antiserum had precipitating antibody detectable at a concentration of antigen as low as 50 ug per ml.

7. Rabbit anti-idiotypic antiserum. This antiserum was prepared in a rabbit to specifically purified IgG anti-SACHO antibody isolated from a single rat (R23G) and containing a cross-specific idiotypic determinant(s) designated as ID1. The rabbit was injected in the hind footpads with 900 ug of isolated R23G light chains mixed with an equal volume of complete Freund's adjuvant. Twenty-five days later, the rabbit was injected subcutaneously in several sites with 3 mg of intact R23G mixed with an equal volume of incomplete Freund's adjuvant. The rabbit was then bled and again boosted with 3 mg of R23G. This reimmunization and bleeding schedule continued at weekly intervals for 3 weeks. The antiserum was absorbed to normal rat IgG antigen excess with pooled normal rat serum and pooled normal rat IgG. The pooled normal rat serum represented equal aliquots (0.2 ml) taken from preimmune sera of 80 rats representing the seven inbred rat strains previously described. The normal rat IgG was isolated by DEAE chromatography from pooled

Sprague-Dawley normal serum. Soluble immune complexes were removed from the absorbed antiserum by chromatography on Sephadex G-200. The absorbed anti-idiotypic antiserum contained 13.3 mg per ml of normal rat IgG as determined by quantitative radial immunodiffusion (Leslie and Swate, 1972).

N-acetyl-D-glucosamine immunoabsorbent. Except for a single modification, an immunoabsorbent for the purification of antibodies to SACHO was prepared as described in detail by Rotta, Krause, Lancefield, Everly and Lackland (1971). The only modification was the use of forty-five mg of p-aminophenyl-B-N-acetylglucosaminide for coupling to 36 ml of CNBr-activated, packed Sepharose 4B (Leslie and Clem, 1973).

Affinity-immunoabsorbent fractionation of anti-SACHO antibodies.

Fractionation of anti-SACHO antibodies was carried out on 0.9 x 29 cm columns containing 5 ml immunoabsorbent (N-acetyl-D-glucosamine) equilibrated in TBS. The immunoabsorbent columns had a binding capacity of 4 mg antibody per 1 ml of packed immunoabsorbent. The antisera applied to the immunoabsorbent column were eluted in three stages: (1) elution of non-immunospecific serum components with 75 ml TBS at 4 C; (2) elution of low-affinity-specific components with 40 ml TBS at 20 C; (3) elution of high-affinity antibodies with an 85 ml gradient at 20 C. The volumes used for the first two stages were empirically

determined, and were always sufficient to effectively separate the non-specific and low-affinity-specific components of the serum samples, as evidenced by final eluate protein concentrations of 8 ug/ml.

Fraction volumes in stages 2 and 3 were 2 ml, and the flow rate for gradient elution was approximately 0.4 ml per min. Protein determinations, based on the optical density at 280 nm utilizing an extinction coefficient of $E_{1\text{ cm}}^{1\%}$, 280 nm = 14.6 for rat immunoglobulin (Binaghi and Benacerraf, 1964), were made on an Acta C III spectrophotometer.

A 500 ml capacity gradient mixer was used for gradient elution. For constant-pH gradients, the starting reservoir contained 42 ml of TBS and the limit reservoir contained an equal volume of the eluting reagent in TBS. Eluting reagents thus used were 3 M NaSCN, 6 M urea, and 15 mM β -N-acetyl-D-glucosamine. In variable-pH gradients, the starting reservoir contained 42 ml saline, pH 7.2, and the limit reservoir contained an equal volume of $\text{CH}_3\text{COOH}/\text{NaCl}$ mixture. The molar range of NaCl was from 0.15 to 1.0; the pH concurrently ranged from 7.2 to 2.4.

Eluates of stages 1 and 2, and antibody fractions from gradient elutions, were collected, dialyzed against TBS, and concentrated to the original serum volume. At this point, measurements of total gradient eluted protein (calculated from the chromatogram) vs. total dialyzed/concentrated protein (undenatured) were made for each of the gradient

elutions to determine the extent of denaturation of the eluted antibodies.

Immunoabsorbent columns were reusable after washing with 20 ml of 6 M urea/0.05 M propionate, followed by 20 ml of TBS. The urea/propionate washing typically removed <1 mg of antibody remaining on the column after completion of the gradient.

PREPARATION OF ANTIBODY FRAGMENTS

1. Rat IgG heavy and light chains. Rat heavy and light chains were prepared by partial reduction of rat IgG anti-SACHO antibody or normal rat IgG in 0.5 M Tris-HCl buffer, pH 8.0 with 0.1 M dithioerythritol at room temperature for 1 hr followed by alkylation with 0.22 M iodoacetamide at 4 C for 1 hr. Extensive reductions were done using the same molarity of dithioerythritol and iodoacetamide but in the presence of either 8 M urea or 7 M guanidine-HCl. The heavy and light chains were isolated by filtration on Sephadex G-200 equilibrated with either 6 M urea + 0.05 M propionic acid or 5 M guanidine-HCl. Of the two distinct peaks resolved, the first contained heavy chains and the second light chains only as determined by Ouchterlony analysis.
2. Rat Fab' fragments and rabbit F(ab')₂ and Fab' fragments. Rat Fab' fragments were prepared by digestion of pooled rat IgG anti-SACHO antibodies with 1% (w/w) pepsin in pH 4.0, 0.1 M acetate buffer at 37 C for 18 hr. After digestion, the fragments were filtered

through a Sephadex G-200 column equilibrated with TBS and calibrated with pooled human IgG and rabbit $F(ab')_2$ and Fab' fragments. The effluent peak corresponding in molecular weight to rabbit Fab' fragments was confirmed by antigenic analysis to contain rat Fab' fragments. Rabbit Fab' fragments were prepared by partial reduction and alkylation of $F(ab')_2$ fragments prepared by 2% (w/w) pepsin digestion of pooled rabbit IgG under the same conditions employed for the rat (Nisonoff, Wissler, Lipman and Woernley, 1960).

Adsorption of nonprecipitating rat anti-SACHO. The IgG fractions obtained by Sephadex G-200 gel filtration of anti-SACHO eluates isolated from the N-acetyl-D-glucosamine immunoabsorbent were selectively adsorbed with GASV and eluted with 5% N-acetylglucosamine. This procedure is described in complete detail elsewhere (Briles and Krause, 1972; Eichmann, Braum, Feizi and Krause, 1970).

Radiolabeling with iodine-125 of specifically purified IgG anti-SACHO antibody containing a cross-specific idiootype (ID1). The procedure for the trace iodination of protein was performed essentially as described elsewhere (McConahey and Dixon, 1966). Specifically purified IgG anti-SACHO antibody (2.5 mg in 2 ml PBS) isolated from a single rat (R55) and containing a cross-specific idiootype hereafter designated as ID1, was labeled with iodine-125 (1 mCi) at 4 C using

chloramine-T (100 ug in 0.5 ml distilled water) as the oxidizing agent. After five minutes the reaction was terminated by adding 100 ug of sodium metabisulfite dissolved in 0.5 ml distilled water. The radiolabeled antibody preparation was fractionated from the unbound iodine by filtration on Sephadex G-25. Precipitation of the radiolabeled antibody with 10% trichloroacetic acid demonstrated >98% precipitable radioactivity.

Radioimmunoassay (RIA) for the detection of ID1 by inhibition of precipitation. RIA for the detection of ID1 was essentially performed as described by Kindt, Seide, Lackland and Thunberg (1972). To a series of Kimble culture tubes (6 x 50 mm) was added 0.1 ml of several dilutions of rabbit anti-idiotypic antiserum in PBS containing 1% bovine serum albumin (PBSA). Then, 0.1 ml containing 0.5 ug of radiolabeled R55G in PBSA (8000-8500 cpm) was added with mixing. The tubes were incubated at 37 C for 2 hr and overnight at 4 C, then centrifuged at 4 C for 20 min at 1000 g to sediment precipitate. Half the supernatant (0.1 ml) was removed and placed in a counting vial. The precipitate tube was placed in a second counting vial and both were counted in a Beckman Biogamma counting system. The amount of precipitated radiolabeled antigen was calculated by using the equation

$$\frac{P-S}{P+S} \times 100 = \text{per cent precipitation}$$

where P equals cpm minus background in precipitate tube and S equals cpm minus background in supernatant tube.

A precipitin curve was constructed and the anti-idiotypic anti-serum dilution which precipitated between 55% and 60% of the radio-labeled antigen was chosen for inhibition of precipitation by the anti-SACHO antisera. To a series of tubes containing 0.1 ml of the appropriate dilution of anti-idiotypic antiserum was added 0.05 ml of anti-SACHO antisera containing 1 to 1.5 ug of anti-SACHO antibody. Control tubes received 0.05 ml of PBSA or pooled normal rat serum. After mixing and incubation for 2 hr at 37 C, radiolabeled R55G (0.1 ml, 0.5 ug) was added and the tubes were incubated and handled as described above. The sensitivity of the RIA in relation to inhibition by unlabeled R55G was demonstrated by constructing an inhibition curve. 0.2 ug of unlabeled R55G produced 23% inhibition of the anti-idiotypic antiserum reacted with 0.5 ug of ^{125}I labeled R55G.

Amino-terminal heavy and light chain sequence of specifically purified IgG anti-SACHO antibodies. Rat IgG anti-SACHO antibodies were specifically purified from the N-acetyl-D-glucosamine immunoabsorbent employing a continuous NaSCN gradient. Heavy and light chains were prepared by either partial or extensive reduction and isolated on Sephadex G-200 equilibrated with 5 M guanidine-HCl. The isolated polypeptide chains

were lyophilized and subsequently sequenced by Dr. J. Donald Capra and Dr. David G. Klapper, the University of Texas, Southwestern Medical School.

RESULTS

ELECTROPHORETICALLY RESTRICTED RAT ANTI-SACHO ANTIBODIES

1. CA electrophoresis of secondary response immune sera:

A careful analysis was initially conducted to define several characteristics of the humoral response to SACHO of adult Sprague-Dawley rats hyperimmunized with GASV. The immune sera of Sprague-Dawley rats hyperimmunized with the streptococcal vaccine will not uncommonly contain antibody fractions that exhibit marked restriction in their electrophoretic mobility. The CA electrophoretic patterns of normal rat serum and the secondary response immune sera from eight different rats, six of which possess restricted protein components in the γ -globulin region (patterns 3-8 inclusive), are shown in Fig. 2A. Presented in the legend is the amount of precipitating antibodies to SACHO in each of the eight antisera. Antisera 1-6 are from F_3 progeny after three selective low-response inbreedings. Antisera 7 and 8 are from F_3 progeny after three selective high-response inbreedings.

2. Determination of restriction in light chain heterogeneity:

To examine more critically the occurrence of antibody restriction, 15 antisera that displayed monodisperse antibody components detected by CA electrophoresis were examined for light chain heterogeneity by alkaline urea polyacrylamide gel disc electrophoresis. Each

Figure 2

A. Cellulose acetate electrophoretic patterns of normal rat serum and the secondary response anti-SACHO antisera from eight different rats.

The amount of precipitating antibody to SACHO (mg/ml) in each antiserum is as follows:

N = normal rat serum	5 = 0.9
1 = 0.5	6 = 5.3
2 = 0.7	7 = 6.5
3 = 0.6	8 = 14.1
4 = 3.7	

B. Polyacrylamide gel disc electrophoretic patterns of extensively reduced and alkylated electrophoretically restricted rat IgG anti-SACHO antibodies.

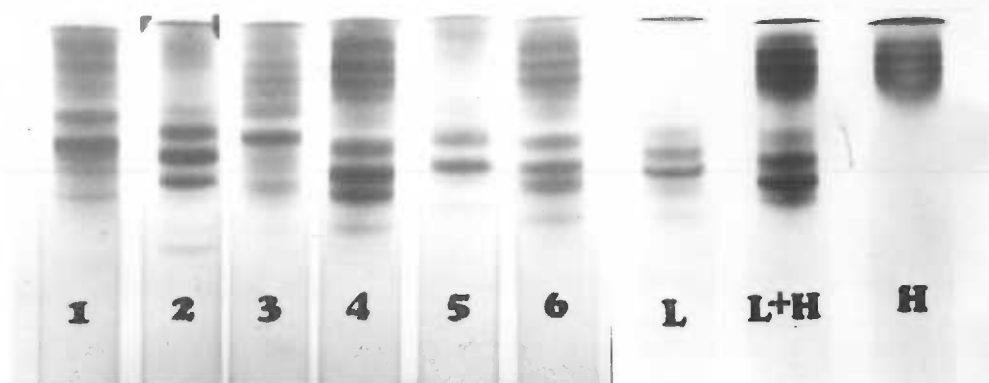
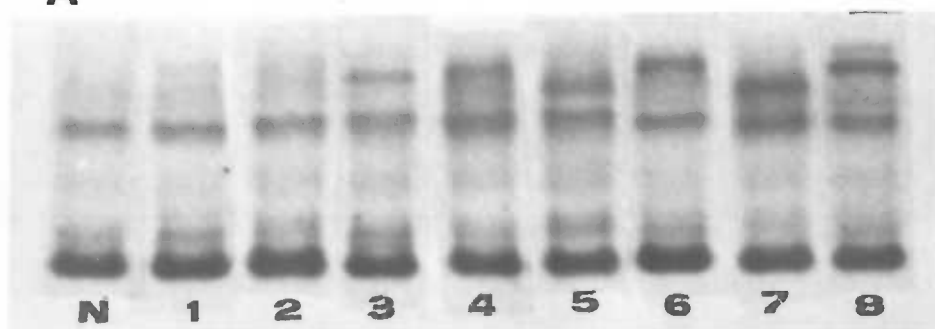
Each gel (1-6) contained 400 ug of extensively reduced IgG antibody. The electrophoretic conditions were the same for each gel, and all were electrophoresed and stained simultaneously. Also pictured are the disc electrophoretic patterns of isolated heavy and light chains from a separate electrophoretically restricted rat IgG anti-SACHO antibody population.

L = isolated light chains

L + H = light plus heavy chains

H = isolated heavy chains

A



B

protein characterized represents the IgG fraction from chromatography on Sephadex G-200 of antibody isolated by elution from N-acetyl-D-glucosamine immunoabsorbents with 2M or 3M sodium thiocyanate. The disc electrophoretic patterns of six representative antisera are shown in Figure 2B (patterns 1-6). Of importance is the fact that the light chains of IgG anti-SACHO from antisera showing restriction by CA electrophoresis were distributed in no more than three major bands, in contrast to normal rat IgG, which displayed at least seven light chain bands of equal intensity (Fig. 8).

Disc electrophoresis of most immune IgG fractions showed distinct banding in the heavy chain region of the gel. This was confirmed by electrophoresis of isolated heavy and light chains chromatographed on Sephadex G-200 equilibrated with 6M urea + 0.05 M propionic acid (Fig. 2B).

It is reasonable to conclude from these observations that monodisperse antibody components detectable by CA electrophoresis represent restriction in light chain heterogeneity.

3. Genetic control of light chain restriction:

The occurrence of light chain restriction appears to be controlled by similar genetic mechanisms in the rat as previously described for the rabbit (Eichmann, Braun and Krause, 1971). For example, the selective inbreeding of brother-sister littermates in which both parents

developed monodisperse antibody components produced six of eight offspring (75%) with monodisperse antibodies. In two cases analyzed, the selective inbreeding of brother and sister littermates, in which one of the parents in each case developed monodisperse antibody components, produced offspring (five of 12 in one case [42%] and four of 11 in another case [37%]) that showed parental-type antibodies of restricted heterogeneity. In contrast, selective breeding of a brother and sister pair showing no restriction produced only one rat in six (16%) with monodisperse antibodies.

4. Recall of electrophoretically restricted antibody:

Five rats with immune sera displaying electrophoretic restriction after secondary immunization were rested for three months, injected on three consecutive days with 0.2 mg of GASV, and bled five days after the last injection. Four of the five antisera contained electrophoretically monodisperse antibody components similar in mobility and magnitude to those seen after secondary immunization. The fifth antiserum contained electrophoretically heterogeneous antibodies.

5. Detection and quantitation of nonprecipitating anti-SACHO:

It was puzzling, however, that concentrations of precipitating anti-SACHO antibody <1 mg per ml (antisera 3 and 5 of Fig. 2A) were capable of producing visible monodisperse segments in the γ -globulin

region. Indeed, preliminary studies occasionally revealed no detectable precipitating antibody in immune sera that possessed restricted protein components subsequently isolated from N-acetyl-D-glucosamine immunoabsorbents.

To eliminate the possibility of nonspecific binding to the immunoabsorbents as an explanation for this discrepancy, 15 ml of normal rat serum was passed over each of two 25-ml portions of immunoabsorbent that were subsequently used for all isolations of antibody. The amount of protein that bound nonspecifically to the immunoabsorbent was <5% of its capacity.

Additionally, protein fractions from five immune sera that were eluted from the immunoabsorbent but contained <10% precipitating anti-SACHO were subsequently passed over Sepharose 4B columns, the insoluble matrix employed in preparation of the N-acetyl-D-glucosamine immunoabsorbent. In every case, <10% of the protein adsorbed to the column. Since most of the additional protein isolated from the immunoabsorbents appeared to be the result of specific binding, the following procedures were used to characterize the eluted protein fractions.

Antisera from all eight rats pictured in Fig. 2A were passed over N-acetyl-D-glucosamine immunoabsorbents, and the adsorbed protein was eluted. From six of the eight antisera (1-6), more protein was eluted from the immunoabsorbent than was expected from quantitative precipitin analysis. In one case (antiserum 5), eight times the amount of protein

was recovered. Fractionation of the eluted protein on Sephadex G-200 revealed distinct differences, confirmed by antigenic analysis, in the relative proportion of IgG to IgM for each of the eight immune sera (Table 1). Because of the relatively small amounts of IgM recovered, these fractions were not further characterized. The IgG fractions from each immune serum, however, were subsequently examined for the presence and quantity of precipitating anti-SACHO, employing RRID in agarose gel.

In testing the IgG fractions from rats 1-6, unusually small precipitin rings appeared when compared with fractions of standard sera containing equivalent amounts of protein present as precipitating antibody. In four antisera (no. 1, 2, 3, and 5), <5% of the eluted and fractionated IgG was present as precipitating antibody, while immune sera from rats 4 and 6 had <20% present as precipitating antibody. In contrast, the IgG fractions from rats 7 and 8 produced precipitin rings that correlated well with the standard samples.

To characterize conclusively the eluted protein fractions as non-precipitating antibody, the IgG fractions from rats 1-6 were selectively adsorbed with GASV and subsequently eluted with 5% N-acetyl-D-glucosamine. In every case, >85% of the IgG adsorbed to the vaccine. Control adsorptions using normal rat IgG demonstrated <10% adsorption.

The total amount of anti-SACHO antibody (nonprecipitating plus precipitating) present per ml in each of the eight immune sera was next

Summary of the secondary immune response of F₃ inbred rats with high- and low-precipitin response to SACHO.

Rat no.	Precipitating (mg/ml)	Nonprecipitating (mg/ml)	Total ^b (mg/ml)	IgG:IgM ^c
1	0.5	0.8	1.3	0.4
2	0.7	0.8	1.5	0.9
3	0.6	3.5	4.1	1.5
4	3.7	3.0	6.7	1.8
5	0.9	6.9	7.8	1.8
6	5.3	6.7	12.0	3.0
7	6.5	0.0	6.5	3.0
8	14.1	0.0	14.1	6.6

Note: Rats no. 1-6 were inbred for a low-precipitin response to SACHO while rats no. 7 and 8 were inbred for a high-precipitin response to SACHO.

^aNonprecipitating antibody equals the difference between total antibody and precipitating antibody.

^bTotal antibody reflects the sum of nonprecipitating plus precipitating antibody as calculated in the text.

^cThe IgG:IgM ratio was calculated on the basis of specifically purified antibody fractionated on Sephadex G-200.

calculated and entered in Table 1. Total antibody levels were determined by subtracting from the amount of protein eluted from the immuno-adsorbent an amount of protein known to adhere nonspecifically to the column. This number was then multiplied by 1.25 which accounts for the denaturation of protein previously calculated to occur in elution of the antibody from the column.

The interpretation of the above findings is that the specifically purified protein components are in fact nonprecipitating antibodies specific for N-acetyl-D-glucosamine. This conclusion is supported by the original isolation of the antibody from specific immuno-adsorbents containing N-acetyl-D-glucosamine covalently bound to Sepharose 4B and the subsequent elution of the antibody from GASV with 5% N-acetyl-D-glucosamine.

AFFINITY-IMMUNOADSORBENT FRACTIONATION OF RAT ANTI-SACHO ANTIBODIES WITH RESTRICTED HETEROGENEITY.

1. CA electrophoresis of rat primary response anti-SACHO antisera:

Rat anti-SACHO antibodies have routinely been purified utilizing the chaotropic ion thiocyanate as a step-wise eluting agent of specific antibodies bound to insoluble immuno-adsorbents. It was of interest, therefore to examine the resolving capability of a continuous thiocyanate gradient employing a number of rat antisera containing electrophoretically restricted anti-SACHO antibodies. More specifically, thiocyanate

was compared with several other eluting reagents which had major disruptive effects on different intermolecular antigen-antibody combining forces.

Ten antisera obtained after a primary immunization series were chosen as representative of the humoral response often seen in Sprague-Dawley rats selectively bred for the occurrence of electrophoretically restricted anti-SACHO antibodies. Fig. 3 contains the CA electrophoretograms and precipitating anti-SACHO antibody levels for these 10 antisera. For comparison, the electrophoretogram of normal rat serum is also shown. The electrophoretically restricted patterns most often displayed only one major band or one major and one minor band in the γ -globulin region of the electrophoretogram. Occasionally, however, two distinct bands were evident (antisera 1 and 10).

2. Comparative elution of anti-SACHO antibodies with thiocyanate and urea gradients:

Anti-SACHO antibodies of the ten antisera were first fractionated from the N-acetyl-D-glucosamine immunoabsorbent by a thiocyanate gradient. The resolving efficiency of thiocyanate gradients was next compared with urea gradients for antisera 1-5.

In general, antisera displaying one major anti-SACHO band (e.g., antisera 3 and 4) were fractionated with equal efficiency by urea and thiocyanate (Fig. 4). The chromatographic patterns displayed

Figure 3

Cellulose acetate electrophoretic patterns of normal rat serum and primary response anti-SACHO antisera of restricted heterogeneity from ten different rats.

The amount of precipitating antibody to SACHO (mg/ml) in each antiserum is as follows:

N = normal rat serum	6 = 13.1
1 = 2.2	7 = 15.8
2 = 28.6	8 = 10.0
3 = 1.4	9 = 15.4
4 = 28.5	10 = 13.7
5 = 23.6	

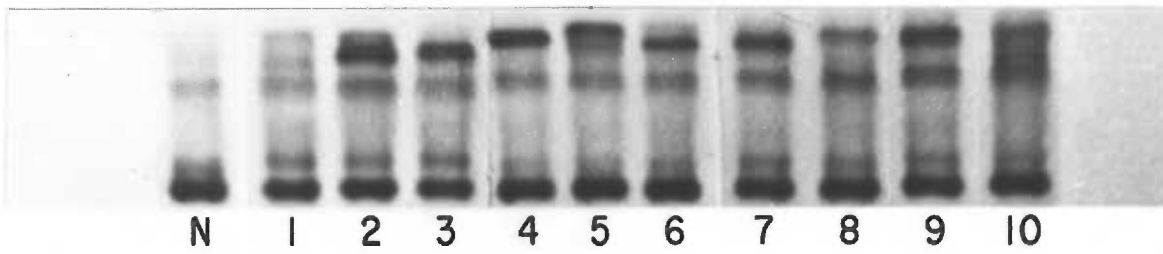
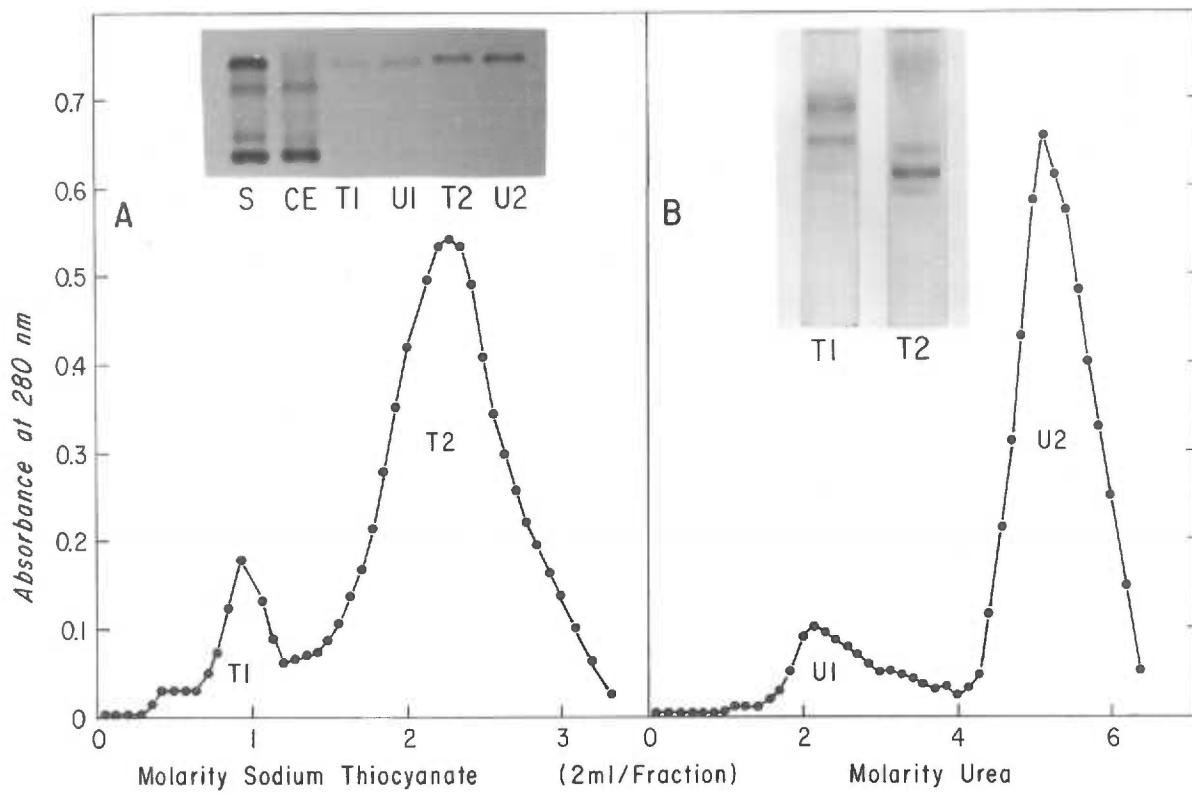


Figure 4

Comparative immunoabsorbent elution by sodium thiocyanate and urea gradients of rat anti-SACHO antibodies with apparent monodisperse electrophoretic mobility.

Chromatographic fractionation of antiserum 4 antibodies from the hapten immunoabsorbent employing (A) sodium thiocyanate and (B) urea gradients. Also pictured as an insert in panel A is the CA electrophoretic analysis of antiserum 4 (S), the non-immunospecific cold elution (CE), sodium thiocyanate chromatographic peaks T1 and T2, and peaks U1 and U2 obtained from the urea gradient. Depicted as an insert in panel B, are polyacrylamide gel disc electrophoretic patterns of partially reduced antibody fractions T1 and T2.



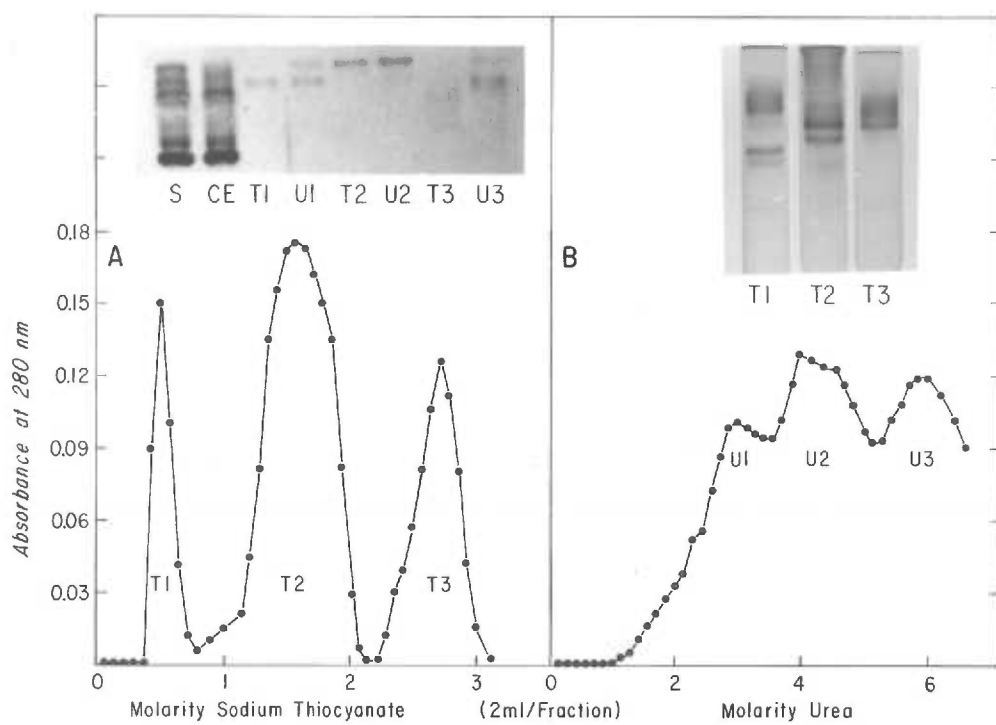
in Fig. 4 were characteristic also of antisera 3, 6, 7, 8 and 9 in that a major antibody population could be isolated by a thiocyanate (or urea) gradient, usually with the degree of purity evidenced electrophoretically (Fig. 4, upper left and right). Of particular significance, however, was the resolution of an apparently single electrophoretic population of antibody molecules present in antiserum 4 into two separate fractions as evidenced by both CA electrophoresis and disc electrophoresis. Both the heavy and light chain banding patterns of the two antibody populations were quite dissimilar. It is concluded, therefore, that antibody molecules of apparently similar net charge may differ widely in both affinity relationships to the immuno-adsorbent column and heavy and light chain banding patterns.

In order to evaluate more critically the affinity-immunoadsorbent fractionating abilities of both thiocyanate and urea, antisera were chosen which displayed at least two electrophoretically distinct anti-SACHO bands. Analysis of two such antisera, antisera 1 and 2, revealed that gradient elution by thiocyanate was superior to urea in resolution of the affinity-specific populations. Fig. 5A documents the thiocyanate fractionation of antiserum 1 antibodies. The efficiency of this one-step fractionation was demonstrated by CA electrophoretic patterns (Fig. 5, upper left) and disc electrophoretic patterns of partially reduced antibody (Fig. 5, upper right). As evidenced in Fig. 5, no consistent correlation could be made between affinity for the

Figure 5

Comparative immunoabsorbent elution by sodium thiocyanate and urea gradients of rat anti-SACHO antibodies with distinct electrophoretic mobility.

Chromatographic fractionation of antiserum 1 antibodies from the hapten immunoabsorbent employing (A) sodium thiocyanate and (B) urea gradients. Also pictured as an insert in panel A is the CA electrophoretic analysis of antiserum 1 (S), the non-immunospecific cold elution (CE), chromatographic peaks T1, T2 and T3 obtained from the sodium thiocyanate gradient, and U1, U2 and U3 obtained from the urea gradient. The insert in panel B depicts the polyacrylamide gel disc electrophoretic patterns of partially reduced antibody fractions T1, T2 and T3. To compensate for the widely differing mobility of heavy and light chains in each antibody fraction, the gels were aligned with reference to the mobility of J chain, represented in each fraction as the fastest migrating (most anodal) protein band.



immunoabsorbent column and electrophoretic mobility of the antibody fractions. For example, the first chromatographic fraction, of lowest relative affinity for the immunoabsorbent column, was electrophoretically faster migrating (more anodal) than the higher affinity second chromatographic peak. The chromatographic fraction of fastest electrophoretic mobility was also of highest relative affinity for the immunoabsorbent column.

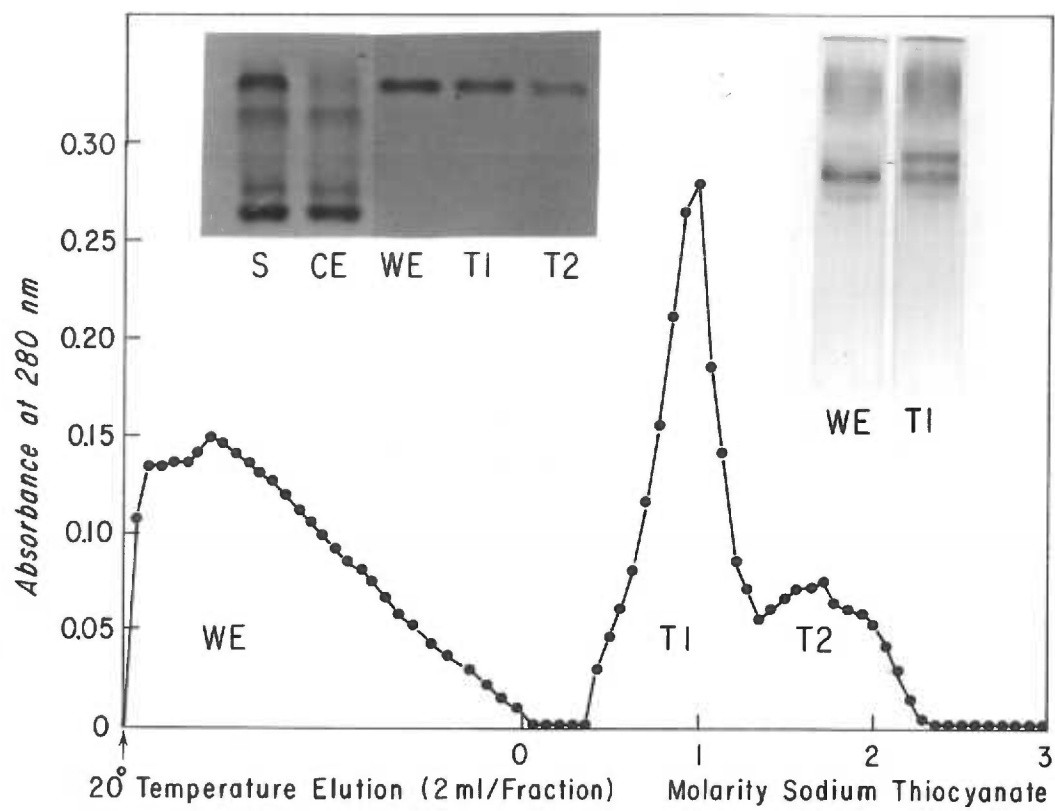
In contrast to the thiocyanate gradient, the urea gradient was less effective in one-step fractionation of antiserum 1, as evidenced both chromatographically (Fig. 5B), and electrophoretically, by the visible degree of adjacent fraction contamination (Fig. 5B, upper left). Barring interfraction contamination, however, the three urea fractions correlated exactly with the thiocyanate fractions in electrophoretic mobility, demonstrating that both gradients fractionated three major antibody populations in the same relative order of affinity for the immunoabsorbent column.

The fractionating ability of thiocyanate was further analyzed, utilizing antiserum 3. A single electrophoretic population of antibodies was resolved into three populations of differing relative affinity for the immunoabsorbent column (Fig. 6). The first and major chromatographic peak was of relatively low affinity for the hapten in that it was eluted from the immunoabsorbent by a 16 C increase in temperature. At a concentration of 10 mg per ml this warm temperature-eluted (WE)

Figure 6

Immunoabsorbent elution with sodium thiocyanate of nonprecipitating rat anti-SACHO antibodies.

Chromatographic fractionation of antiserum 3 antibodies from the hapten immunoabsorbent employing a sodium thiocyanate gradient. A major population of low affinity antibodies was eluted from the immunoabsorbent by a 20 C increase in temperature (WE). Also pictured is the CA electrophoretogram of antiserum 3 (S), the non-immunospecific cold elution (CE), the low-affinity warm temperature eluted fraction (WE), and chromatographic fractions T1 and T2 obtained from the sodium thiocyanate gradient (upper left). Upper right depicts the polyacrylamide gel disc electrophoretic patterns of partially reduced antibody fractions WE and T1.



fraction contained undetectable precipitating antibody to SACHO. Since the remaining nine antisera contained <1 mg per ml of WE antibody and as gradient-eluted antibody was always shown to be precipitating, it might be inferred that nonprecipitating anti-SACHO antibody is antibody of low affinity for the immunoabsorbent.

CA electrophoretic analysis of antiserum 3 revealed identical electrophoretic mobilities and therefore identical net charge for both major chromatographic peaks although these fractions differed in relative affinity for the immunoabsorbent column. Disc electrophoresis, however, confirmed differences between the light chains of both fractions (Fig. 6, upper right).

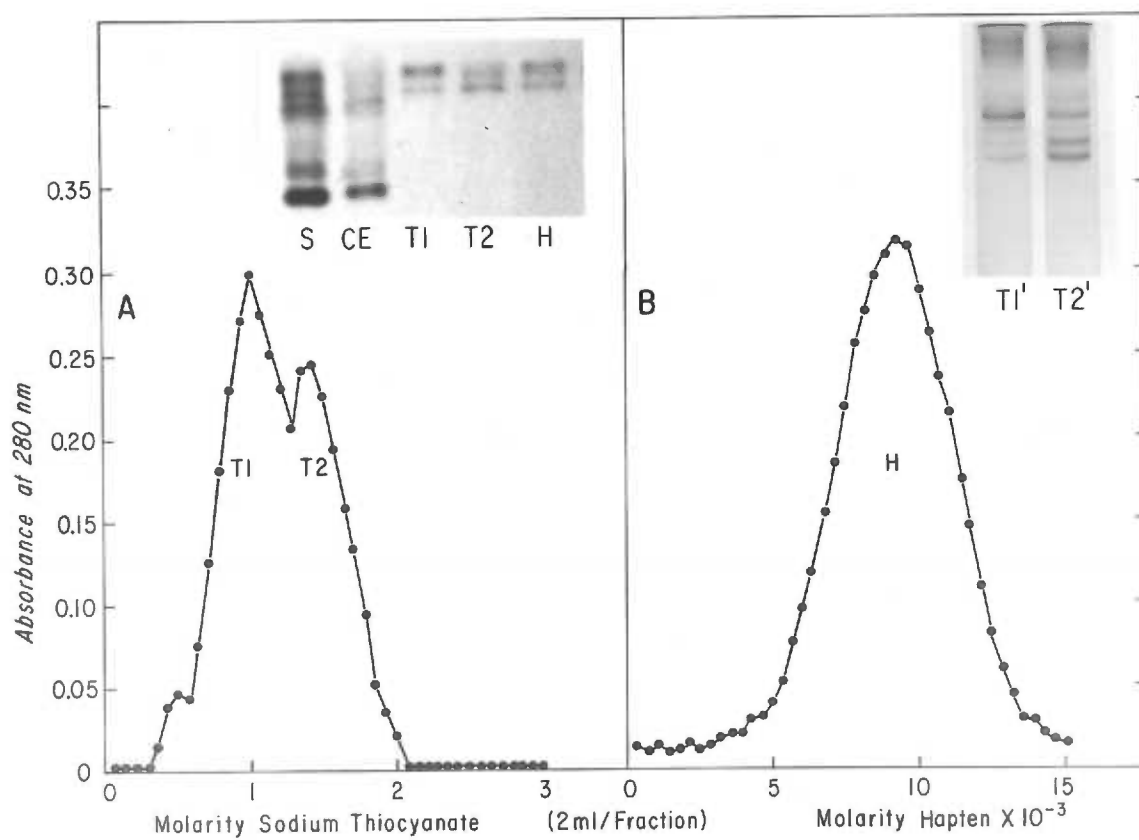
3. Comparative elution of anti-SACHO antibodies with thiocyanate and hapten gradients:

In order to correlate strict immunospecificity with affinity-immunoabsorbent fractionation, antibodies from antisera 2 and 10 were fractionated with a hapten gradient (N-acetyl-D-glucosamine). In both cases, chromatographic and electrophoretic criteria demonstrated the superiority of thiocyanate over hapten in separating antibody populations of differing net charge. Two major antibody populations of very similar relative affinity for the immunoabsorbent column were partially separable by the thiocyanate gradient. (Fig. 7A) whereas the hapten gradient totally failed to separate these two affinity populations

Figure 7

Comparative immunoadsorbent elution by sodium thiocyanate and hapten gradients of rat anti-SACHO antibodies with distinct electrophoretic mobility.

Chromatographic fractionation of antiserum 10 antibodies from the hapten immunoadsorbent employing (A) sodium thiocyanate and (B) hapten (N-acetyl-D-glucosamine). Also pictured is the CA electrophoretic analysis of antiserum 10 (S), the non-immunospecific cold elution (CE), chromatographic peaks T1 and T2 obtained from the sodium thiocyanate gradient, and the single hapten gradient peak (H) (upper left). Upper right depicts the polyacrylamide gel disc electrophoretic patterns of partially reduced antibody fractions T1' and T2', the rechromatographed peaks obtained from the second sodium thiocyanate gradient.



(Fig. 7B). The two affinity populations demonstrated in Fig. 7A were isolated by refractionation of each chromatographic peak on the same immunoadsorbent column employing a second thiocyanate gradient.

Examination of T1' and T2', the rechromatographed peaks, demonstrated distinct mobilities by CA electrophoresis but very similar heavy and light chain banding patterns by disc electrophoresis (Fig. 7, upper right). It is concluded, therefore, that anti-SACHO antibodies which differ in electrophoretic mobility by CA electrophoresis and thereby possess different net charge may exhibit similar heavy and light chain banding patterns and have similar relative affinity for the immunoadsorbent column.

4. Comparative elution of anti-SACHO antibodies with thiocyanate and acid-salt gradients:

Finally, in the interest of establishing whether or not there might be functionally important charged groups located in the active site of the antibodies in this system, and therefore titratable below conformationally disturbing pH values, several experiments were conducted on antisera 9 and 10, utilizing various acetic acid/sodium chloride gradients. Antiserum 9 remained bound to the immunoadsorbent despite an environment of pH 3.6 and 1 M NaCl. Below pH 3.6 all antibody of antiserum 9 was eluted as one peak. Antiserum 10 was eluted as one sharp peak below pH 4.0. In addition, a gradient of 6 M urea/

0.05 M propionic acid failed to elute antiserum 9 as effectively as the thiocyanate gradient.

5. Comparison of denaturation caused by elution reagents:

In developing a practical and effective fractionation procedure, one realizes the importance of the choice of a minimally denaturing elution reagent. Although the degree of denaturation increased with increasing molarity of the eluting agent, in general, denaturation by either thiocyanate or urea was <20%, while hapten denaturation was minimal.

GENETIC INFLUENCES ON THE MAGNITUDE OF THE PRECIPITIN RESPONSE OF RATS TO SACHO

1. High-precipitin response accompanied by restriction in light chain heterogeneity:

Rats immunized with GASV may produce high levels of precipitating anti-SACHO antibody often associated with electrophoretic restriction of the corresponding antibody and its constituent light chains. The most dramatic humoral response observed to date is represented by the secondary response antiserum of an F₄ rat bred for a high-precipitin response to SACHO. This individual rat (R23) produced 40 mg/ml of precipitating antibody to the group A specific carbohydrate. CA electrophoresis of the antiserum suggested a highly restricted response

to SACHO. This observation was confirmed by the disc electrophoretic pattern of isolated light chains from specifically purified IgG anti-SACHO antibody (Fig. 8). Approximately 90% of the light chains were localized to one major band. Normal rat IgG light chains routinely produced seven bands of approximately equal intensity. It is proposed that the strikingly elevated anti-SACHO precipitin response exemplified by this animal is a direct result of selective inbreeding. Evidence supporting this assumption is provided by the following lines of investigation.

2. Selective inbreeding of high- and low-precipitin responders:

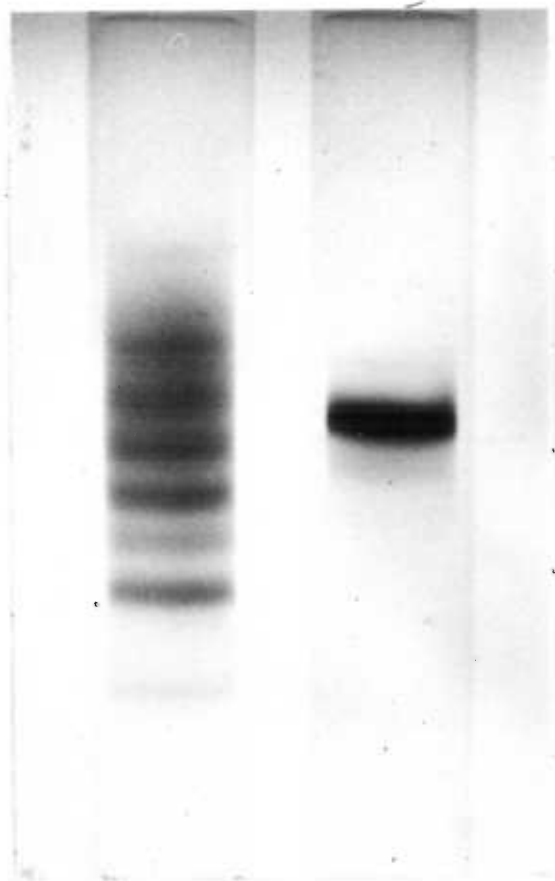
To evaluate a possible genetic influence on the magnitude of the precipitin response of rats to SACHO, a series of brother-sister inbreedings were undertaken in an attempt to segregate high-precipitin responders (HPR) from low-precipitin responders (LPR).

Early in the course of inbreeding (F_2), it was observed that animals bred for a low-precipitin response had lost the ability to enhance their precipitin response after a secondary series of immunizations. These animals were quite capable, however, of mounting a primary precipitin response that often exceeded the magnitude of that observed in rats bred for a high-precipitin response. Continued generations of brother-sister inbreedings were therefore necessary to lower the primary precipitin response of animals bred for a low-precipitin response.

Figure 8

Polyacrylamide gel disc electrophoresis of isolated light chains from normal rat IgG and specifically purified IgG anti-SACHO antibody from an F₄ rat bred for a high-precipitin response to SACHO.

Polyacrylamide gel disc electrophoresis of isolated light chains from normal rat IgG (NIgG) and specifically purified IgG anti-SACHO antibody from an F₄ rat bred for a high-precipitin response to SACHO (R23G). The electrophoretic conditions were the same for each gel and both were electrophoresed and stained simultaneously.



NIgG R23G

After four generations of selective breeding, a family of HPR was segregated from a family of LPR. These families were segregated in the sense that the highest precipitin response of the LPR was lower than the lowest secondary precipitin response of HPR. Indeed test bleedings from rats bred for a low-precipitin response after primary (week 3) and each week of secondary immunization (weeks 4, 5, 6) revealed that 8 of 11 rats (73%) produced <1 mg per ml of precipitating antibody throughout immunization. In contrast, 5 of 10 (50%) F₄ rats bred for a high-precipitin response produced >10 mg per ml of precipitating antibody.

Fig. 9 compares the precipitating anti-SACHO levels of the F₄ LPR family with those of the F₄ HPR family. Data for the preceding parental generations are also given. It is evident that the secondary precipitin response of the HPR is often three or more times the primary precipitin response. In contrast, no LPR was capable of mounting a significant secondary precipitin response.

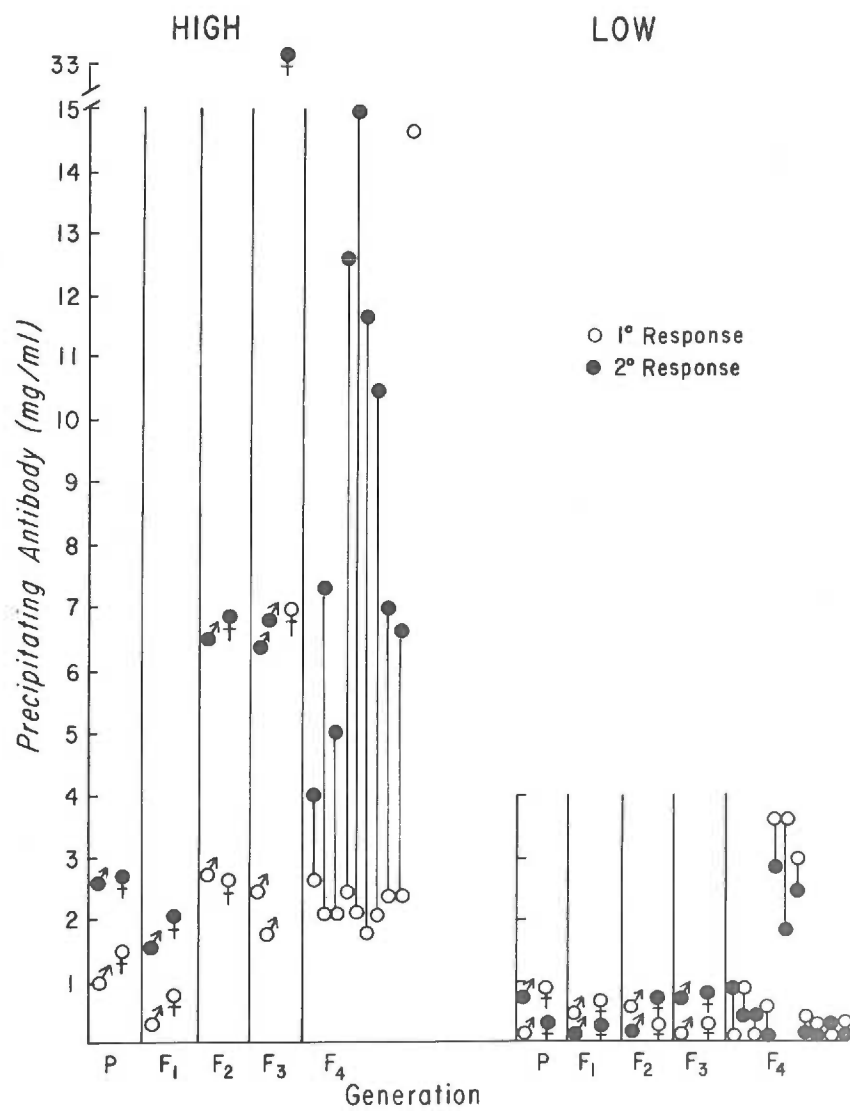
3. Prolonged immunization of low responders:

To examine the ability of LPR to maintain or alter their response under prolonged immunization, 5 rats bred for a low-precipitin response were immunized with GASV for a total of 18 weeks. Following primary and secondary immunization (6 weeks), all rats were immunized with one intravenous injection weekly of 0.2 mg of GASV for 9 weeks. this course of immunization was followed by three weekly intravenous injections,

Figure 9

Anti-SACHO precipitin response of F_4 progeny selectively bred for high- and low-precipitin response to SACHO.

Serum concentration of anti-SACHO precipitins in F_4 progeny derived from selected high- and low-responding breeding pairs. Precipitin levels of parents selected from each generation are also pictured. Open circles O = precipitin concentrations after primary immunization. Closed circles ● = precipitin concentrations after secondary immunization. One animal in the F_4 high precipitin response generation died after primary immunization.



on consecutive days, of 0.2 mg for 3 weeks. All blood collections were made 5 days after the last injection of each week. All 5 rats showed peak precipitin levels at the end of primary immunization (week 3; Fig. 10). It is concluded, therefore, that prolonged immunization of low responding rats, under these conditions, fails to enhance the precipitin response; thereby lending further support to the importance of genetic factors on the precipitin response of low responders.

4. Variation of antigen dose:

To further analyze the secondary response of LPR, one family of F_5 LPR was immunized with the standard 0.2 mg dose of vaccine during secondary immunization. A second family of F_5 LPR was immunized with 1 mg of vaccine during secondary immunization. Both families remained LPR, with no difference in the secondary precipitin response between them (Table 2). It appears therefore, that the inability of LPR to mount a secondary precipitin response greater than the primary precipitin response is not a function of the dose of antigen.

5. Antibody specificity:

Since β -N-acetyl glucosaminidase is present in rat liver and kidney (Pugh, Leaback and Walker, 1957; Sellinger, Beaufay, Jacques, Doyen and DeDuve, 1960), it was of interest to examine anti-SACHO antisera for streptococcal group A-variant carbohydrate antibody. Group A-variant

Figure 10

Anti-SACHO precipitin levels during prolonged immunization of five rats selectively bred for a low-precipitin response to SACHO.

Serum concentration of anti-SACHO precipitins during prolonged immunization of five rats selectively inbred for a low-precipitin response to SACHO. The immunization schedule is described in the text. The first bleeding (week 3) represents the end of primary immunization.

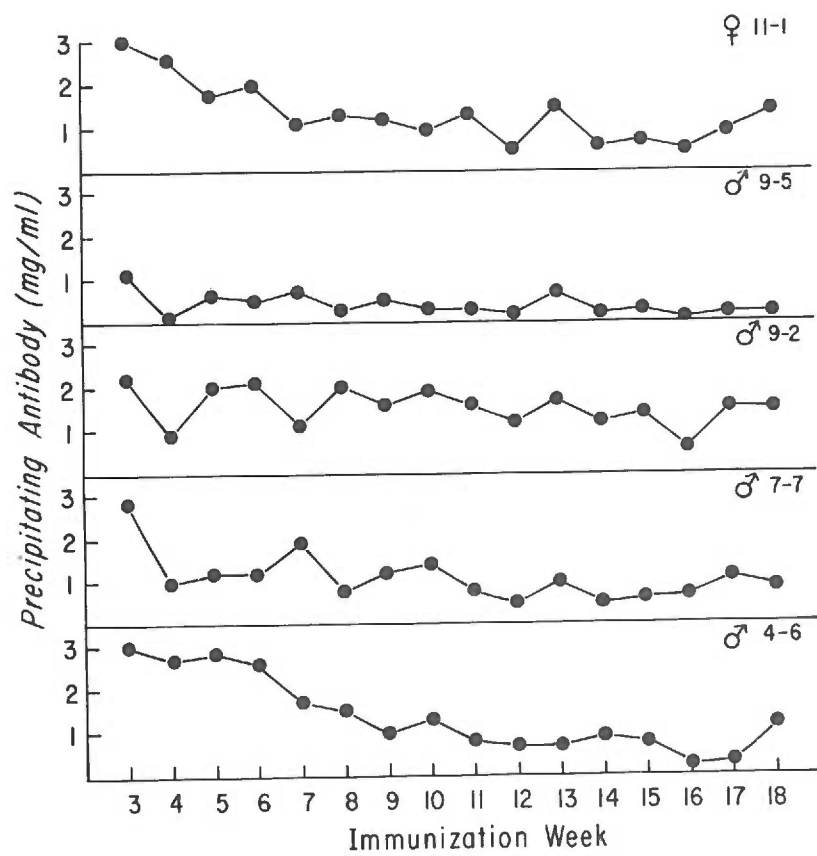


Table 2

Effect of antigen dose on secondary response of F₅ low-precipitin responders.^a

<u>Antigen dose^b : 0.2 mg</u>			<u>Antigen dose^b : 1.0 mg</u>		
Animal no.	Precipitins (mg/ml)		Animal no.	Precipitins (mg/ml)	
	1° response	2° response		1° response	2° response
1-1	0.5	0.2	2-1	1.2	1.5
1-2	2.5	2.1	2-2	1.4	1.7
1-3	2.3	2.6	2-3	0.8	0.6
1-4	0.4	0.7	2-4	0.9	0.8
1-5	0.5	0.3	2-5	0.4	0.3
1-6	0.6	0.9	2-6	0.1	0.4
1-7	0.7	1.0	2-7	1.0	0.6
1-8	0.9	0.5	2-8	0.5	0.5

^aAll animals in both groups received three weekly injections of 1 mg of vaccine on consecutive days for 3 weeks during primary immunization.

^bAntigen dose administered on 3 consecutive days weekly for 3 weeks during secondary immunization.

carbohydrate lacks the terminal N-acetyl glucosaminide residues of group A carbohydrate and has its serological specificity in rhamnose-rhamnose linkages (McCarty, 1956). In only one antiserum from 88 high- and low-responding rats, representing the first three generations of inbreeding, could detectable concentrations of precipitating anti-A-variant antibody be demonstrated. Therefore, unlike mice that also possess β -N-acetyl glucosaminidase, these rats do not appear to alter the native antigenic configuration of SACHO (Schwab and Brown, 1968). Whether this is a function of antigen dose, route of immunization, or the intrinsic properties of the rat enzyme is yet to be determined.

RAT INTERSTRAIN ANTIBODY RESPONSE TO SACHO

In view of numerous reports demonstrating the association or linkage of histocompatibility loci with immune response genes to a wide variety of antigens, recently reviewed by Gasser and Silvers (1974), an interstrain analysis of the precipitin response of rats to SACHO was conducted.

1. Serum concentration of anti-SACHO precipitins in seven inbred rat strains hyperimmunized with GASV:

Male and female rats from each of seven inbred strains representing six major rat histocompatibility haplotypes, Ag-B (Palm and Black, 1971) or H-1 (Stark, Kren and Gunther, 1971) were hyperimmunized with GASV and

bled five days after the last injection of each week. The immunization schedule was identical to that administered for the establishment of Sprague-Dawley LPR and HPR. Quantitation of anti-SACHO precipitin levels was made for each weekly bleeding. The highest percentage of animals responding from each strain occurred at the end of secondary immunization (week 6). Presented in Table 3 is the mean secondary anti-SACHO precipitin response of the seven inbred rat strains. For comparison, the mean precipitin response of F_7 Sprague-Dawley rats selectively bred for a low-precipitin response to SACHO (F_7 SDLPR) and the mean precipitin response of F_6 Sprague-Dawley rats selectively bred for a high-precipitin response to SACHO (F_6 SDHPR) are also listed. The low-precipitin response of all inbred rat strains was quite similar to the F_7 low responding Sprague-Dawley rats and in striking contrast to the F_6 high responding Sprague-Dawley rats. Although these data do not conclusively discount an association or linkage of the major rat histocompatibility locus with immune response genes to SACHO, they suggest that the Ag-B locus may not be involved. Nevertheless, under the conditions employed for immunization, the observations emphasize the success of selective high-response inbreeding in establishing a rat strain of high-precipitin responders to SACHO.

2. Cellulose acetate electrophoretic analysis of anti-SACHO antisera from seven inbred rat strains:

Qualitative analysis of the anti-SACHO antisera obtained following

Table 3

Anti-SACHO precipitin response of inbred rat strains.

Rat strain	No. animals immunized	Ag-B haplotype (histocompatibility)	Mean precipitin ^a response (mg/ml)	Range (mg/ml)	% responding ^b
F344	12	1	0.2	0.0-0.8	42
W/Fu	11	2	0.5	0.0-1.5	90
BN	11	3	0.1	0.0-0.3	55
Cop	16	4	0.2	0.0-0.5	81
Aug	11	5	0.4	0.3-0.6	100
M520	8	6	0.4	0.0-1.7	38
Buf	9	6	0.1	0.0-0.9	22
F ₇ SDLPR	37	- ^c	0.2	0.0-1.0	43
F ₆ SDHPR	8	- ^c	16.0	5.0-35.6	100

^aArithmetic mean of secondary precipitin response.^bLower level of detection of antibody is 65 ug/ml.^cUndetermined.

each week of immunization was carried out to determine the occurrence in each rat strain of anti-SACHO antibodies with restricted heterogeneity. Although the low-precipitin response in these strains made it difficult to visualize restricted antibody components, in general, it was possible to conclude that electrophoretically restricted antibodies were present in highest frequency at the end of secondary immunization (week 6).

Interstrain comparison of the CA electrophoretograms at week 6 demonstrated nine of eleven (82%) of Aug anti-SACHO antisera which contained electrophoretically restricted antibody components. Six of the nine restricted antisera had one predominant antibody band while three antisera contained two distinct antibody components. In contrast to Aug rats, no more than three antisera from each of the other rat strains possessed electrophoretically restricted antibodies. Therefore, with the immunization schedule employed, although strain differences are not apparent in the magnitude of the precipitin response to SACHO, the qualitative expression of anti-SACHO antibodies of restricted heterogeneity is more frequently observed in Aug rats than the six other inbred rat strains examined.

CROSS-IDIOTYPIC SPECIFICITY OF RAT ANTI-SACHO ANTIBODIES.

The ability to elicit and isolate anti-SACHO antibodies with molecular uniformity, in association with genetic influences on the antibody response, allows the procurement of anti-idiotypic antisera and subsequent analysis of idiotype determinants on rat anti-SACHO antibodies.

1. Electrophoretic and Ouchterlony analysis of idiotypic and anti-idiotypic antibodies:

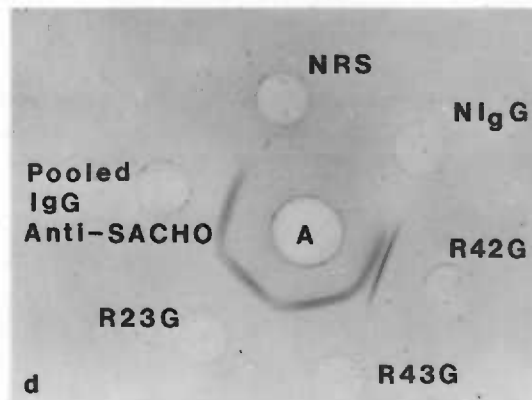
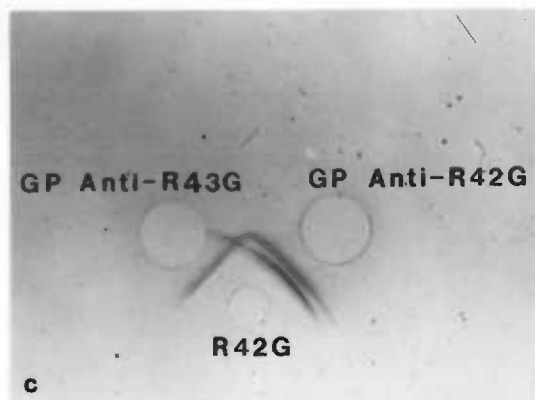
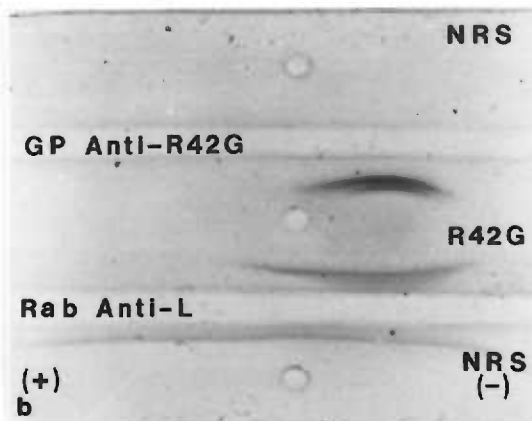
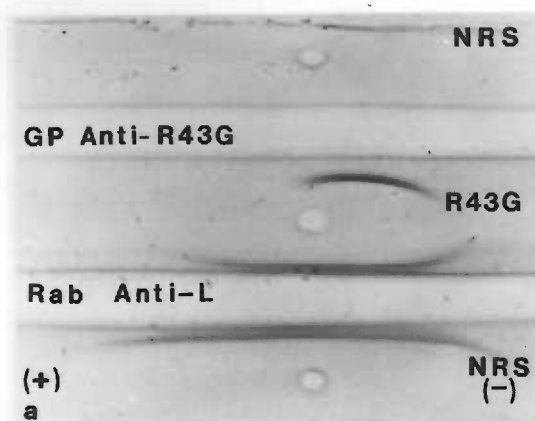
IgG antibodies from two rats, R42 and R43, with anti-SACHO precipitin levels of 5.5 and 14.1 mg per ml, respectively, were utilized to elicit anti-idiotypic antibodies in guinea pigs. Immune sera from both rats showed the presence of electrophoretically restricted antibody components first demonstrated by CA electrophoresis and subsequently confirmed by polyacrylamide gel disc electrophoresis of the reduced and alkylated IgG anti-SACHO antibodies. By disc electrophoresis, R42 IgG antibody (R42G) displayed two major light chain bands while R43 IgG antibody (R43G) had only one major light chain band. Pooled rat IgG routinely produced seven light chain bands of approximately equal intensity (Fig. 8).

Immunoelectrophoretic analysis of both R42G and R43G against the corresponding guinea pig antisera absorbed with pooled normal rat serum revealed one precipitin line for R43G (Fig. 11a) and two precipitin lines for R42G (Fig. 11b) all in the IgG region of the electrophoretogram. The lines were shorter, however, than those obtained in the reaction of the same antibody preparations with rabbit anti-rat light chain antiserum. These results confirm reports of other investigators (Braun and Krause, 1968; Bokisch, Chiao and Bernstein, 1973) and demonstrate the limited electrophoretic heterogeneity of the subpopulation of antibody molecules that reacts with the absorbed guinea pig antisera.

Figure 11

Immuno-electrophoretic and Ouchterlony analysis of guinea pig anti-idiotypic antisera.

- a. Immuno-electrophoretic analysis of guinea pig anti-R43G (GP anti-R43G) and rabbit anti-rat light chain antisera (Rab anti-L) reacted against pooled normal rat serum (NRS) and R43G.
- b. Immuno-electrophoretic analysis of guinea pig anti-R42G (GP anti-R42G) and rabbit anti-rat light chain antisera (Rab anti-L) reacted with pooled normal rat serum (NRS) and R42G.
- c. Ouchterlony analysis of both guinea pig anti-idiotypic antisera (GP anti-R43G; GP anti-R42G) reacted with R42G.
- d. Ouchterlony analysis of guinea pig anti-R42G (A), reacted with pooled normal rat serum (NRS), pooled normal rat IgG (NIgG), representative IgG anti-SACHO antibodies (R42G, R43G, R23G) and pooled rat IgG anti-SACHO.



For further characterization of the specificity of the absorbed guinea pig antisera, anti-R43G and anti-R42G were reacted against R42G by double diffusion in agarose gel (Fig. 11c). Of the two distinct precipitin lines formed between R42G and anti-R42G, one was identical and the second non-identical with the single precipitin line formed between R42G and anti-R43G. This result suggests the common idio type (ID1) detected by both antisera and in the case of anti-R42G, its ability to detect a distinct idio type (ID2).

2. Inhibition of idiotypic:anti-idiotypic reaction:

The incorporation of N-acetyl-D-glucosamine or D-glucosamine hydrochloride into the agarose at concentrations as low as 0.05 M and 0.15 M, respectively, completely inhibited the reaction of anti-R42G and anti-R43G with their corresponding immunogens at concentrations of 1 mg per ml. The homologous reaction of bovine anti-rat IgG with both R42G and R43G, however, was not inhibited. Slightly higher concentrations of N-acetyl-D-glucosamine (0.1 M) and D-glucosamine hydrochloride (0.2 M), however, were necessary to inhibit the precipitin reaction of 1 mg/ml of either R42G or R43G with 100 ug per ml SACHO. Inhibition of the idiotypic:anti-idiotypic reaction at a lower concentration of sugar than required for antibody-SACHO inhibition may reflect the fact that not all of the antibody molecules in either R42G or R43G combine with the anti-idiotypic antisera, although all can combine with SACHO.

Rhamnose hydrate or anhydrous D-glucose at a concentration of 0.3 M did not inhibit either the idiotypic:anti-idiotypic reaction or the precipitin reaction of 1 mg per ml R42G or R43G with 100 ug per ml SACHO. The ability of hapten to inhibit the idiotypic:anti-idiotypic precipitin reaction clearly implicates the antigen-binding site or an area adjacent to the binding site in determining the specificity of these idiotypic determinants.

3. Occurrence of shared idiotypic determinants among unrelated animals:

To evaluate the occurrence of these idiotypic determinants among unrelated individuals, anti-SACHO antisera from 25 outbred Sprague-Dawley rats were analyzed by double diffusion in agarose gel against both absorbed guinea pig antisera. Nineteen of the 25 antisera formed a single precipitin line against both anti-R42G and anti-R43G, all of which showed complete identity with the single precipitin line formed between R43G and both absorbed guinea pig antisera (ID1). It was of interest therefore to examine the antibody fractions of anti-SACHO antisera from five selectively inbred rat families. Twenty-five specifically purified IgG anti-SACHO antibody fractions, including a pool from approximately 60 animals, were analyzed as described above (Table 4). These antibodies were used as antigens at a concentration of 1 mg per ml. Seventeen of the 25 preparations, including the pool, formed a single precipitin line against anti-R42G antiserum all of which showed complete

Table 4

Occurrence of ID1 on specifically purified IgG anti-SACHO antibodies isolated from selectively inbred rat families.^a

Family	No. examined	No. positive
A	6	4
B	3	2
C	5	4
D	5	3
E	5	3

^aPresence of ID1 in pooled anti-SACHO IgG not listed.

identity with the first of the two R42G:anti-R42G precipitin lines (Fig. 11d). This precipitin line was also identical to that formed between anti-R43G and R42G and therefore corresponds to ID1 as previously described.

Aliquots of anti-R42G were absorbed with one of the 17 IgG antibody fractions which reacted with both anti-R42G and anti-R43G (R81G). After absorption, anti-R42G only reacted with its immunogen (R42G) and not with any of the same 25 antibody preparations previously tested. This loss of reactivity confirms the antigenic identity of the determinants shared by the 17 distinct antibody populations.

These idiotypic specificities could not be detected in the sera of these 17 rats before immunization in those cases where this could be tested nor in the antisera after adsorption on N-acetyl-D-glucosamine immunoabsorbents. The removal of idiotypic activity by immunoabsorption provides strong evidence that the reaction with anti-idiotypic is idiotypic and not allotypic since the allotype of the purified antibody should be represented on antibody molecules other than those with anti-SACHO activity. In like manner, the reaction with anti-idiotypic does not appear to be isotypic, i.e., selection by SACHO and enrichment of a minor subclass of rat IgG. First, this subclass would have to be present in normal rat serum at a concentration <50 μg per ml and second, SACHO would have to select from within the subclass to explain the restricted light chain heterogeneity. Additional evidence against an

isotype:anti-isotype reaction occurring is provided by the localization of ID1.

4. Localization of ID1 on the antibody molecule:

Under routine conditions of pepsin digestion, pooled rat IgG anti-SACHO antibodies containing ID1 were split predominately into Fab' fragments. The univalency of these fragments, which corresponded in molecular weight to rabbit Fab' fragments, was demonstrated by their inability to precipitate with SACHO but retention of the capacity to bind to N-acetyl-D-glucosamine immunoabsorbents. Reaction of these fragments, which bound to specific immunoabsorbents, with anti-R42G and anti-R43G revealed single lines of precipitation which were identical with the single precipitin line formed between these antisera and the undigested rat IgG anti-SACHO antibodies. This initial localization of ID1 is in agreement with results by other investigators who have shown that idiotypic determinants are found on the Fab fragments of IgG molecules (Braun and Kelus, 1973; Grey, Mannik and Kunkel, 1965).

In an attempt to restrict further the localization of ID1, heavy and light chains were prepared by partial reduction of rat IgG anti-SACHO antibody containing ID1 (R23G). Reaction of isolated heavy and light chains against anti-R42G and anti-R43G revealed single lines of precipitation between the light chain preparation and both absorbed guinea pig antisera. These precipitin lines were identical with the

single precipitin line formed between the anti-idiotypic antisera and the unreduced antibody fraction (R23G). No reaction was observed with the heavy chain preparation.

These observations suggest that ID1 represents antigenic determinants on the light chains of anti-SACHO antibodies. The presence of ID1 on anti-SACHO light chains promotes the argument that the cross-specificity observed in this study may be due to λ light chain isotypic determinants. Recent observations with immunoglobulin-producing tumors in rats belonging to the LOU-Wsl strain confirm the low frequency of λ light chains on rat immunoglobulins (Querinjean, Bazin, Starace, Beckers, Deckers and Heremans, 1973). It is conceivable, therefore, that SACHO may select and enrich for clones of anti-SACHO cells which secrete antibody with λ light chains.

5. Non-identity of ID1 with λ light chain isotypic determinants:

The interspecies cross-reactivity of a chicken anti-human λ light chain antiserum was utilized to evaluate rat anti-SACHO antibodies for the occurrence of λ light chains and their antigenic identity or non-identity with ID1. Fifteen specifically purified IgG anti-SACHO antibody preparations containing ID1, examined at concentrations ranging from 0.1 mg per ml to 10 mg per ml, were reacted with the chicken anti-human λ light chain antiserum by Ouchterlony. None of the fifteen antibody fractions precipitated with the chicken antiserum specific for

λ light chains. This finding is in contrast to the detection of λ light chains by this chicken antiserum in pooled normal rat serum and demonstrates both the non-association and more important the non-identity of ID1 with λ light chain isotypic determinants. Additionally, three separate specifically purified IgG anti-SACHO antibody preparations containing ID1 were analyzed for the presence of κ or λ light chains. All three antibody preparations reacted strongly with an anti-rat κ light chain antiserum and showed no reactivity with an anti-rat λ light chain antiserum (J. D. Capra and D. G. Klapper, personal communication).

6. Stability in expression of ID1:

In view of the extensive cross-specificity of ID1, the expression of this common idotype would be expected to be a stable gene product. To examine this possibility, the occurrence of ID1 was examined in two F_4 rat families selectively inbred for a high- and low-precipitin response to SACHO (Fig. 9). Nine of the eleven (82%) F_4 LPR and eight of the ten (80%) F_4 HPR possessed antigenically identical idiotypic determinants, ID1, as demonstrated by Ouchterlony analysis. Therefore, a selectively bred rat family which produced <1 mg per ml of anti-SACHO antibody, qualitatively expresses ID1 in the same frequency as animals from a family producing >10 mg per ml anti-SACHO. It would appear that the qualitative expression of ID1 is not associated with the magnitude of the anti-SACHO precipitin response and represents a stable gene product.

7. Specificity of rabbit anti-idiotypic antiserum:

To extend further the analysis of ID1 employing RIA, a rabbit anti-idiotypic antiserum was prepared by immunization with specifically purified IgG anti-SACHO antibody (R23G). This antibody, R23G, reacted strongly with both guinea pig anti-idiotypic antisera and provided therefore an antigenic stimulus for the production of anti-ID1 (Fig. 11d).

Analysis by Ouchterlony of the appropriately absorbed rabbit anti-idiotypic antiserum (anti-R23G) demonstrated a reaction of partial identity to the guinea pig anti-idiotypic antiserum (anti-R43G) when reacted against R23G (Fig. 12a). This finding suggests additional idiotypic determinants present on R23G which are detectable by anti-R23G but undetectable by anti-R43G.

Subsequent analysis of anti-R23G by Ouchterlony employing the same 25 specifically purified antibody preparations previously tested for reactivity with anti-R43G (Table 4) demonstrated positive precipitin reactions for the same 17 antibody preparations previously shown to contain ID1. These reactions were, however, only of partial identity to the reaction of anti-R23G and R23G.

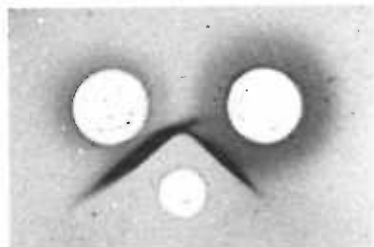
Anti-R23G was next reacted by Ouchterlony with separate fractions of R23 anti-SACHO antibody isolated from the N-acetyl glucosamine immunoabsorbent by a discontinuous sodium thiocyanate gradient (Fig. 12b). Reaction of anti-R23G with a 0.5 M sodium thiocyanate eluate and a 2 M sodium thiocyanate eluate (following a 1 M elution) demonstrated reactions

Figure 12

Ouchterlony analysis of absorbed rabbit anti-idiotypic antiserum.

- A. Ouchterlony analysis of rabbit anti-R23G (Rab anti-R23G) and guinea pig anti-R43G (GP anti-R43G) reacted with R23G.
- B. Ouchterlony analysis of rabbit anti-R23G (A), reacted against R23G isolated heavy chains (R23G-H); a 0.5 M NaSCN eluate of R23 anti-SACHO antibody (0.5 M R23); R23G; a 2 M NaSCN eluate of R23 anti-SACHO antibody and R23G isolated light chains (R23G-L).

Rab Anti-R23G GP Anti-R43G



R23G

A

R23G-H

R23G

0.5MR23



R23G-L

R23G

2MR23

B

of identity with R23 anti-SACHO antibody. As expected, reaction of isolated heavy and light chains from R23G with anti-R23G demonstrated a positive precipitin reaction of identity between the light chains and the intact IgG antibody. No reaction was observed with the heavy chain preparation. This finding is in agreement with earlier observations employing guinea pig anti-idiotypic antisera and demonstrates the localization of ID1 to the light chains of anti-SACHO antibodies. Moreover, the above observations suggest the presence of antigenically identical idiotypic determinants on distinct anti-SACHO antibodies isolated from the same antiserum (R23).

Earlier observations on cross-idiotypic specificity of rat anti-SACHO antibodies employed double diffusion in agarose gel as the assay for detection of ID1. In contrast to this qualitative determination, more sensitive methods were necessary to detect cross-specific idiotypic determinants on rabbit anti-streptococcal Group C carbohydrate antibodies (Kindt, Seide, Bokisch and Krause, 1973). This distinguishing feature in the detection of idiotypic cross-specificity on rat and rabbit anti-streptococcal carbohydrate antibodies may reflect the predominance in expression of a single idio type on rat anti-SACHO antibodies in contrast to the minor expression of a cross-specific idio type on rabbit anti-streptococcal antibodies. In reference to the latter, the observations of Klapper and Kindt (1974) indicate that idiotypic

cross-reactions in rabbit anti-streptococcal antisera are due to a minor antibody component in each reactive antiserum.

If in fact a common idiotype gene is predominantly selected and expressed on rat anti-SACHO antibodies, its gene product would be expected in all rat strains examined. Confirmation of the occurrence of ID1 in other rat strains would also provide additional evidence for the non-association of ID1 with any single rat allotypic determinant. With this in mind, therefore, the cross-specificity of ID1 on rat anti-SACHO antibodies from nine inbred rat strains was investigated.

8. Preliminary interstrain detection of ID1 by double diffusion in agarose gel:

From a pilot study on the anti-SACHO response of eight inbred rat strains (F344, Lew, W/Fu, ACI, Cop, Aug, Buf and M520) (G. A. Leslie, unpublished data) selective antisera were available to analyze the interstrain occurrence of ID1. Employing double diffusion in agarose gel, ID1 was detected in anti-SACHO antisera from all eight inbred rat strains. However, because in some strains antisera from only a few animals were analyzed and since most preimmunization sera were not available, an extensive analysis of ID1 was conducted on rat anti-SACHO antisera from the seven inbred rat strains listed in Table 3.

9. Detection of ID1 by inhibition of precipitation employing RIA:

A sensitive RIA was developed for the detection of ID1 in order to delineate the quantitative expression of ID1 among the seven inbred rat strains. The rabbit anti-idiotypic antiserum employed in the RIA was absorbed to an antigen excess of 13.3 mg per ml of normal rat IgG. Accounting for the dilution of the anti-idiotypic antiserum used in the RIA, a 2000-fold excess of normal IgG in relation to the radiolabeled idiotypic antibody (R55G, 0.5 ug) was present in each tube determination. Each anti-SACHO antiserum was diluted and used as an inhibitor in the RIA at a concentration of 1.0 to 1.5 ug of precipitating anti-SACHO antibody. For 75% of the antisera this represented a 1:10 dilution. This level of antibody was selected for inhibition to demonstrate the predominant expression of ID1 since the use of unlabeled R55G at a concentration of 1 ug produced 87% inhibition of the idiotypic:anti-idiotypic reaction. Preimmunization sera from each animal were also used as inhibitors either at a 1:5 or 1:10 dilution.

The results of the inhibition assay employing anti-SACHO antisera representing seven different inbred rat strains, six major rat histocompatibility haplotypes determined by the B locus and both major rat allotypic light chain determinants (Armerding, 1971) are shown in Table 5. All inbred rat strains examined demonstrated a remarkably high percentage of ID1 cross-specificity. In contrast, all preimmunization sera from these animals were negative for ID1 (<5% inhibition). The

Table 5

Interstrain occurrence of ID1 in rat anti-SACHO antisera.^a

Rat strain	No. antisera examined	Ag-B haplotype (histocompatibility)	Allotype W-1 SD-1	% positive	Range ^b % inhibition	Mean ^b % inhibition
F344	6	1	-	100	20-100	65
W/Fu	10	2	+	40	24-75	53
BN	6	3	+	50	16-55	37
Cop	10	4	NA ^c	50	14-81	54
Aug	10	5	+	30	20-36	29
Buf	4	6	+	75	57-100	71
M520	6	6	NA ^c	83	76-100	92
F ₇ SDLPR	10	-	-	100	50-100	90

^aAll preimmunization sera from these animals were negative for ID1 (<5% inhibition).^b% inhibition of ID1 positive antisera.^cNA denotes not available.

remarkable stability in expression of ID1 is further demonstrated by its presence in all analyzed anti-SACHO antisera obtained from F₇ Sprague-Dawley rats selectively inbred for a low-precipitin response to SACHO (F₇SDLPR).

Since in every case, however, only antisera obtained at the end of secondary or primary immunization were examined, no comparison can be made between the relative percent occurrence of ID1 in each inbred rat strain.

10. Analysis of rat anti-SACHO IgG isolated heavy and light chains for the occurrence of ID1 employing RIA:

Since previous observations employing Ouchterlony, apparently localized ID1 to the light chains only of anti-SACHO antibodies, isolated heavy and light chains from four ID1 positive antibodies were used as inhibitors in the RIA. As shown in Table 6, all four intact IgG antibodies demonstrated marked inhibition when used as inhibitors at concentrations ranging from 1.0 to 3.5 ug. Except for the isolated light chains obtained from R23G, however, only slight inhibition occurred when isolated heavy or light chains from the three additional antibodies were used as inhibitors at relatively high concentrations. Although at first, these observations might appear contradictory, both to each other and to the original localization of ID1 employing Ouchterlony, a more careful analysis explains the apparent discrepancy. In reference to the method

Table 6

RIA inhibition analysis of heavy and light chains isolated from ID1 positive IgG anti-SACHO antibodies.

<u>Inhibitor</u>	<u>ug added</u>	<u>% inhibition</u>
R23 IgG anti-SACHO	3.5	55
R23 IgG anti-SACHO light chains	7.2	41
R23 IgG anti-SACHO heavy chains	22	39
R55 IgG anti-SACHO	1	87
R55 IgG anti-SACHO light chains	11	18
R55 IgG anti-SACHO heavy chains	3	0
R51 IgG anti-SACHO	3	79
R51 IgG anti-SACHO light chains	27	22
R51 IgG anti-SACHO heavy chains	30	18
Pooled IgG anti-SACHO	3	58
Pooled IgG anti-SACHO light chains	30	15
Pooled IgG anti-SACHO heavy chains	43	18

for preparation of the anti-idiotypic antiserum, the rabbit was initially immunized with isolated light chains from R23G and subsequently boosted with intact R23G. Therefore, a mixture of antibodies specific for both idiotypic determinants associated solely with the light chains and those requiring heavy and light chain interaction might be expected. Indeed, isolated light chains prepared from R23G reacted less strongly by the method of Ouchterlony with the anti-idiotypic antiserum than did the intact molecule (Fig. 12). Moreover, a single precipitin line would be expected when the rabbit antiserum was reacted with R23G even if two antibody populations were present in the anti-idiotypic antiserum. The original observation by the method of Ouchterlony, of weak reactivity of R23G isolated light chains was subsequently confirmed by RIA. Intact R23G demonstrated marked inhibition of the idiotypic:anti-idiotypic reaction at a lower concentration than did R23G isolated light chains. Taken together, then, the observations by Ouchterlony and RIA methods demonstrate that although a major part of the idiotypic determinant(s) designated as ID1 is present on the light chain, the complete expression of the entire determinant requires heavy and light chain interaction. The low level of inhibition of isolated heavy chains examined as inhibitors in the RIA would support this assumption.

11. Interspecies analysis of ID1:

In view of the extensive rat interstrain cross-specificity of ID1

on anti-SACHO antibodies, anti-SACHO antisera obtained from rabbits, goats and chickens and specifically purified antibodies from nurse sharks were analyzed for the occurrence of ID1. Twenty chicken anti-SACHO antisera were used as inhibitors in the RIA at concentrations of anti-SACHO antibody ranging from 10 ug to 60 ug. Fifteen of the chicken antisera were used at concentrations of anti-SACHO antibody greater than 25 ug. None of the twenty chicken antisera demonstrated inhibition (<5%). Similarly, 10 rabbit and 2 goat anti-SACHO antisera, at concentrations of antibody as high as 40 ug, also showed lack of inhibition. Finally, specifically purified anti-SACHO antibody from 11 nurse sharks, used as inhibitors at a concentration of 50 ug, failed to demonstrate inhibition of the idiotypic:anti-idiotypic reaction. These findings indicate that ID1 is a species-specific idio type and are similar to observations on mouse anti-SACHO cross-idiotypic specificity (Briles and Krause, 1974).

12. Interantigenic analysis of ID1 on rat antibodies from the same donor antisera:

To examine the occurrence of ID1 on rat antibodies to an antigen unrelated to SACHO, poly-GA, 14 selectively bred F₇ Sprague-Dawley rats including both high- and low-responders to SACHO, were sequentially immunized with poly-GA and GASV. The results of an RIA inhibition analysis of anti-poly-GA antisera and anti-SACHO antisera obtained from

each animal are presented in Table 7. None of the 14 anti-poly-GA antisera demonstrated inhibition of the idiotypic:anti-idiotypic reaction (<5%). In striking contrast, after immunization with GASV, all anti-SACHO antisera obtained from these same 14 animals demonstrated marked inhibition. It should be noted that the anti-poly-GA antisera were used as inhibitors at concentrations of anti-poly-GA at least as high as the anti-SACHO antibodies. Moreover, preimmunization sera obtained from all animals demonstrated no inhibition (<5%). This finding suggests the necessity for specific antigenic challenge with GASV and a resultant anti-SACHO response for the expression of ID1.

13. Amino-terminal heavy chain sequence analysis of rat anti-SACHO antibodies containing ID1:

In view of the molecular restriction and extensive cross-idiotypic specificity of rat anti-SACHO antibodies, it was of interest to sequence the amino-terminal portion of isolated anti-SACHO heavy chains. Heavy chains were prepared and isolated from ID1 positive IgG anti-SACHO antibodies obtained from three separate antisera by affinity-immuno-adsorbent chromatography. As shown in Figure 13, the amino-terminal heavy chain sequence of all three antibodies was quite similar to that of the human V_H III subgroup for the first 30 amino acids. However, identical amino acid substitutions distinct from the V_H III subgroup, occurred at positions 3, 16, 20, and 28 in all three heavy chain

Interantigenic analysis of ID1 on antibodies of different specificity obtained from the same donor sera.

Animal no. ^a	Anti-poly-GA (ug) ^b	% inhibition	Anti-SACHO (ug) ^b	% inhibition
1	7.0	0	3.5	100
2	5.0	0	3.5	100
3	8.0	0	2.0	100
4	4.0	0	3.5	100
5	5.0	0	2.0	56
6	5.0	0	5.0	100
7	5.0	0	6.0	96
8	10.0	0	1.0	63
9	6.0	0	1.5	100
10	13.0	0	2.5	38
11	12.0	0	5.0	83
12	8.0	0	4.0	65
13	19.0	0	7.5	100
14	2.0	0	3.5	79

^aRats no. 1-9 were inbred for a low response to SACHO while rats no. 10-14 were inbred for a high response to SACHO.

^bAmount of anti-poly-GA or anti-SACHO antibody used for inhibition.

Figure 13

Amino-terminal heavy chain sequence of idiotypically-related rat anti-SACHO antibodies.

Rat IgG pool sequence obtained from LOU strain rats.

Rat anti-SACHO sequence represents identical sequence obtained from three separate IgG antibody populations (R42G, R34G and R55G) containing ID1.

R34G Rest sequence was obtained from IgG isolated from rat R34 two months following secondary immunization with GASV.

** represents a maximum of 10% lysine.

Note: Solid line represents sequence identity with Human V_{HIII} subgroup.

5 10 15 20 25 30

Human V_H III

GluValGlnLeuValGluSerGlyGlyGlyLeuValGlnProGlyGlySerLeuArgLeuSerCysAlaAlaSerGlyPheThrPheSer

Rat IgG Pool

Lys

Rat Anti-SACHO

Lys Arg LysIso Asp

R34G (Rest)

** Lys

preparations. That these four substitutions are in fact a result of specific antigenic challenge with GASV and a resultant anti-SACHO antibody response is evidenced by the lack of these four substitutions in IgG obtained from one of these same three animals (R34G) two months following immunization with GASV. The low level of lysine at position 3 in R34G Rest, most likely reflects the presence of residual anti-SACHO antibody in this IgG preparation. It is concluded from the above observations, that idiotypically-related rat antibodies to SACHO, are associated with a unique heavy chain variable region subgroup.

DISCUSSION

Hyperimmunization of Sprague-Dawley rats with a heat-killed, pepsin-treated vaccine of group A streptococci elicits specific anti-carbohydrate antibodies of restricted heterogeneity. The presence of monodisperse antibody components in the immune response of rats to SACHO is not unlike the response of rabbits (Braun, Eichmann and Krause, 1969) and is apparently controlled by similar genetic mechanisms (Eichmann, Braun and Krause, 1971).

It is significant that restriction in light chain heterogeneity, confirmed by polyacrylamide gel disc electrophoresis of specifically purified antibody, could be demonstrated in the rat with minimal purification of antibodies. Preparative electrophoresis of the antibody samples might have reduced further the number of light chains detected by disc gel electrophoresis (Eichmann, Lackland, Hood and Krause, 1970). Moreover, these monodisperse antibody components were capable of recall after extended periods of nonimmunization.

Previous explanations for a uniform antibody response to antigenic stimulation (Chen, Strosberg and Haber, 1973) include:

- 1) the restricted response may be due to an antigen of very restricted structure which stimulates a small number of antibody producing clones.
- 2) the ability to produce antibodies of restricted heterogeneity

may be the result of genetic factors.

3) the appearance of clones of antibody producing cells with high affinity for antigen may sequester the antigen from other low affinity clones and produce a restricted response.

4) the hyperimmunization with antigen may provide a toleragenic stimulus which represses most clones, leaving only a few to proliferate and produce restricted antibody.

In relating these possible explanations to observations obtained with the humoral response of rats to SACHO, each mechanism by itself is inadequate in explaining the generation of antibodies with restricted heterogeneity. For example, in reference to the restricted nature of the antigenic stimulus, a range of response to SACHO from heterogeneous to monodisperse is observed upon immunization with GASV of an outbred population of Sprague-Dawley rats. This is consistent with observations in rabbits immunized with streptococcal and pneumococcal vaccines. Indeed, Chen, Strosberg and Haber (1973) reported heterogeneous, restricted and monoclonal responses sequentially seen in the same animal.

Overgrowth of a clone producing higher affinity antibody which sequesters antigen from lower affinity clones is also inconsistent with present observations. For example, some rats may respond with specific antibodies of restricted heterogeneity which possess unusually low affinity for an immunoadsorbent column (Fig. 6). The inability of these

specifically purified antibodies to precipitate with antigen also suggests antibodies of low affinity for antigen.

Although conclusive data cannot be drawn from the response of rats to SACHO to eliminate selective tolerance as an adequate explanation for the generation of antibodies with restricted heterogeneity, observations on the response of rabbits to pneumococcal vaccine demonstrate the failure of this theory to account for the continual emergence of new antibody producing clones during immunization (Chen, Strosberg and Haber, 1973).

Finally, observations in the rat (Stankus and Leslie, 1974a) and rabbit (Eichmann, Braun and Krause, 1971; Pincus, Mage, Alexander and Chace, 1973) confirm the influence of genetic factors on the degree of restriction of antibodies to streptococcal and pneumococcal carbohydrates. However, the initial observations by Chen, Strosberg and Haber (1973) demonstrate that nearly all rabbits in an outbred population can respond with restricted and often monoclonal responses to pneumococcal carbohydrate at one time or another during prolonged immunization.

It is apparent therefore that an interplay of mechanisms is in operation in determining the degree of antibody restriction in response to carbohydrate antigens.

The induction by streptococcal vaccines of specific nonprecipitating antibodies is exemplified in the humoral response of the rabbit to group A-variant vaccine (Braun, Eichmann and Krause, 1969). By use of

representative antisera from rats bred for either a high- or low-precipitin response to SACHO, nonprecipitating antibody was isolated from the immune sera of all F_3 animals bred for a low-precipitin response. It is of interest to note that rats bred for a low-precipitin response, which produced moderate levels of precipitating antibody (antisera 4 and 6 of Fig. 2), also had detectable levels of nonprecipitating antibody. This is in striking contrast to rats bred for a high-precipitin response, in which nonprecipitating antibody was negligible. Antisera 4 and 6 demonstrate the fact that after three selective low-response inbreedings not all offspring have completely segregated into low-precipitin responders.

Certain characteristics of the antibody response that distinguish F_3 high from low inbred responders are summarized in Table 1. The eight animals analyzed, corresponding to the eight cellulose acetate electrophoretograms pictured in Fig. 2A, are representative of >40 animals thus far similarly studied. In addition to the presence of nonprecipitating antibody, the chromatographic ratios of IgG to IgM are lower in rats bred for a low-precipitin response than for rats bred for a high-precipitin response. This conclusion is based on an estimate of the total amount of antibody (nonprecipitating plus precipitating) present per ml in each of the eight immune sera. This observed difference in the IgG to IgM ratio (antiserum 4 compared with antiserum 7 and antiserum 6 compared with antiserum 8) may simply be due to a carry-over

of IgM from the primary response. The carry-over of IgM may reflect the inability of a subpopulation of cells actively secreting antibody to shift from production of IgM to production of IgG or may in fact represent a regulatory mechanism similar to that described in strains of non-responder mice (Ordal and Grumet, 1973).

The isolation from antisera of monodisperse or electrophoretically restricted antibodies has generally employed affinity-immunoadsorbent chromatography (Eichmann and Greenblatt, 1971; Cheng, Fraser and Haber, 1973). It was of interest, therefore, to develop a simple, effective technique of affinity-immunoadsorbent chromatographic fractionation of rat antibodies to SACHO which exhibited electrophoretic restriction. The term affinity is used with the qualification that a measurement is made of the functional affinity (avidity) of the antibodies for an immunoadsorbent. Evaluation of several eluting reagents including sodium thiocyanate, urea, N-acetyl-D-glucosamine and acid-salt demonstrated the chaotropic ion thiocyanate to be most effective in separating rat anti-SACHO antibody populations of individual affinity characteristics.

The effects of thiocyanate (and chaotropic ions in general) on antigen-antibody interactions have previously been discussed by Dandliker, Alonso, de Saussure, Kierszenbaum, Levison and Schapiro (1967). They mention four probable effects: 1) reversing hydrophobic interactions possibly by altering the water of hydration and allowing unfolding or

dissociating processes to take place; 2) electrostatic shielding or weakening of the chemical interaction between charged groups; 3) the salting out effect; and 4) ion binding, leading to protein unfolding or dissociation. Dandliker, Alonso, de Saussure, Kierszenbaum, Levison and Schapiro (1967) were the first to report utilizing thiocyanate for immunospecific purification. They achieved higher, though not necessarily maximal, recovery of precipitable antibody from serum with 2 M thiocyanate (65%) than with other chaotropic reagents: 2 M perchlorate (36%) and 2 M iodide (25%).

In contrast to thiocyanate, urea exerts its disruptive effects on proteins principally through breaking intrachain hydrogen bonding. As the C=O and NH₂ groups in the urea molecule are structurally very similar to the CO and NH portions of the peptide linkage, then urea, especially at high concentrations, displaces peptide-originating intrachain hydrogen bonding. Hydrogen bonding, however, is probably not the major factor in conferring stability to antigen-antibody complexes. As Singer (1965) pointed out, the water molecule is such a strong hydrogen bond donor and acceptor, that it competes successfully for the acceptor and donor groups of the solute.

Affinity-immunoabsorbent chromatography utilizing 6 M urea was first described by Bata, Gyenes and Sehon (1964). Although they reported a low yield of antibody (20%) as compared with elution by a glycine-HCl buffer at pH 3, the low denaturing effect of urea on protein,

which had been reported, might ultimately compensate for the limited effect it seemed to have. However, under the employed experimental conditions, a urea gradient never separated anti-SACHO antibody populations of restricted heterogeneity better than a thiocyanate gradient. The superior fractionating ability of thiocyanate in comparison to urea is best evidenced by Fig. 5.

In general, under acid and alkaline conditions, 7S immunoglobulins undergo conformational changes. Singer (1965) states that 7S immunoglobulins do not exhibit any detectable change in molecular conformation between pH 4 and 9, but outside this range, they do. Affinity-immuno-adsorbent fractionation in the SACHO:anti-SACHO system by acid alone, however, appeared to be theoretically unworkable. The hapten was the electrically neutral (at pH 7.2) hapten β -N-acetyl-D-glucosamine and it was unlikely that charged groups (e.g. carboxyl) would play an important role in intermolecular bonding.

As is the case with acid, it was difficult to conceptualize how a salt gradient (in effect, salting out) could exert an affinity-specific effect on separating antibodies by their affinity characteristics. In this antigen-antibody system, the antibodies are effectively out of solution to begin with, since they are bound to an insoluble matrix. Nevertheless, several experiments were conducted utilizing various acetic acid/sodium chloride gradients in an attempt to fractionate rat anti-SACHO antibodies with restricted heterogeneity. In every case,

the antibodies were eluted in a single peak from the immunoabsorbent in contrast to the resolution into distinct fractions by a thiocyanate gradient.

The conclusion is therefore drawn that hydrophobic interactions are of primary importance in determining the intermolecular binding forces in this antigen-antibody system. This conclusion rests on the superior fractionating ability of thiocyanate, a chaotropic ion which preferentially disrupts these bonds. At the same time, the data support a secondary role for hydrogen bonding, whereas it offers no evidence for significant electrostatic interactions.

Affinity-immunoabsorbent fractionation of anti-SACHO antibodies by a thiocyanate gradient allowed a comparison between functional affinity of these antibodies for the immunoabsorbent column and corresponding electrophoretic mobility. In general, no consistent overall relationship was found between functional affinity (avidity) and electrophoretic mobility as judged by either CA electrophoresis or disc electrophoresis. Fig. 5 best exemplifies this conclusion. This finding was not altogether unexpected since several chromatographic peaks of the ten primary response antisera contained both IgM and IgG anti-SACHO antibodies as evidenced by immunoelectrophoresis and Ouchterlony analysis. In addition, antiserum 2 contained IgA anti-SACHO antibodies.

Gradient elution with thiocyanate established the necessity of examining both net charge and charge distribution in analyzing affinity

relationships between antibodies. For example, it was found that antibodies migrating the same as judged by CA electrophoresis and therefore exhibiting the same net charge, possessed widely differing affinity characteristics for the immunoabsorbent column (Fig. 6). Indeed, the population of antibodies of lowest affinity for the immunoabsorbent (WE) failed to precipitate with SACHO while higher affinity antibody (T1) was found to contain precipitating antibodies to SACHO. These antibody fractions (WE and T1) were further found to differ in their light chain bonding pattern. The occurrence of low affinity, non-precipitating antibody in antiserum 3 was initially suggested by the major discrepancy between the CA electrophoretogram and quantitative precipitin analysis (Fig. 3). In addition, this particular animal originated from a cross-mating of parents also possessing nonprecipitating anti-SACHO antibodies.

In contrast to antiserum 3, antiserum 10 (Fig. 7) is an example of two distinct populations of anti-SACHO antibodies, which differ in both affinity for the immunoabsorbent column and net charge, as judged by CA electrophoresis, yet possess similar heavy and light chain banding patterns.

It is concluded, therefore, that an analysis of affinity-immunoabsorbent relationships of antigen-antibody reactions should take into consideration the following: 1) dissociating agent used; 2) class of antibodies present; 3) net charge of antibody populations; and

4) distribution of charge among the heavy and light chains of corresponding antibodies.

Preliminary investigation of the precipitin response of the rat to SACHO suggested a genetic influence on the magnitude of the response (Leslie and Carwile, 1973). This hypothesis was confirmed when a family of HPR was segregated from a family of LPR after four selective brother-sister inbreedings.

A number of factors are important in determining the fewest number of generations necessary to achieve segregation of response to a particular antigen (McDevitt and Benacerraf, 1969). Some of these influences are, however, operational in a sense and therefore deserve to be mentioned. For example, many times during the course of inbreeding, low litter numbers, infertility, abnormal sex ratios among littermates, or cannibalism of offspring necessitated the mating of two less desirable animals. These influences on achieving segregation are exemplified by the undesirably chosen F_1 parents of HPR and the necessity for mating two males with the same female to produce a reasonable litter size in the F_4 generation of HPR (Fig. 9).

The data presented (Fig. 9) represent one family bred for a low-precipitin response to SACHO and a separate family bred for a high-precipitin response to SACHO. These particular families were chosen for comparison simply because complete data were available for both parents and progeny during all four generations of inbreeding. It should be

stressed, however, that other families bred for either a low- or high-precipitin response showed responses similar to those of the families described. Undoubtedly the most apparent similarity among rats bred for a low-precipitin response was the inability to mount a secondary precipitin response of greater magnitude than the primary precipitin response.

A sometimes unexamined variable in analyzing the segregation of humoral immunologic responsiveness is the occurrence of nonprecipitating antibody. Previous studies have confirmed the presence of significant concentrations of nonprecipitating anti-SACHO antibodies in rats selectively bred for a low-precipitin response to SACHO (Stankus and Leslie, 1974a). A simple comparison, which suggests the presence of elevated levels of nonprecipitating antibody, is the correlation of the cellulose acetate electrophoretogram with the quantitative precipitin analysis of each immune serum. This determination was made for all of the F_4 LPR, and no major discrepancies could be found between the electrophoretograms and quantitative precipitin analysis. It would seem therefore that these animals are in fact "true" low responders to SACHO.

Additional evidence implying a genetic influence on the precipitin response arises from the prolonged immunization and variation in the dose of antigen during secondary immunization of LPR. That the precipitin response of LPR could not be enhanced by varying both the dose of antigen and the frequency of challenge clearly suggests a genetic

predisposition for a low-precipitin response to SACHO.

It was indeed striking that such a small percentage of rats respond to GASV by producing precipitins to group A-variant carbohydrate. Since β -N-acetyl glucosaminidase is localized in the liver and other organs of the rat, it was necessary to examine antibody specificity because conceivable cleavage of N-acetyl glucosamine from the rhamnose backbone on GASV might induce group A-variant precipitins. From the present findings it is apparent that the structure of group A carbohydrate either does not change to one with A-variant specificity to any extent or the change to A-variant structure with a resultant anti-A-variant precipitin response occurred after the blood samples were collected.

Genetic control of the immune response of rats to a wide variety of antigens has been well documented (Simonian, Gill and Gershoff, 1968; Gill and Kunz, 1971; Günther, Rüde and Stark, 1972; Würzburg, Schütt-Gerowitt and Rajewsky, 1973). Further experiments have shown that rat immune response genes to lactic dehydrogenase isoenzyme A are functionally expressed in thymus-derived lymphocytes only (Günther, Rüde and Stark, 1972). Although the data presented does not localize the genetic influences on rat anti-SACHO response to a particular level in the immune response (macrophage, B cell, T cell), it is conceivable that these genetic influences are also thymus-dependent, as previously demonstrated in the mouse (Braun, Kindred and Jacobson, 1972).

The association or linkage of immune response genes with major rat histocompatibility loci has largely been demonstrated employing synthetic polymers of amino acids as the antigenic stimulus (Günther, Rüdé and Stark, 1972; Simonian, Gill and Gershoff, 1968). In general, the antibody response of high-responding strains has been measured in μ g amounts. In contrast to the humoral response of rats to synthetic peptides, however, the response of selectively bred rats to SACHO may reach antibody levels as high as 40 mg per ml (Stankus and Leslie, 1975). In the interest of establishing whether or not certain inbred rat strains might likewise produce high levels of anti-SACHO, and with the hope of correlating the response to the major rat histocompatibility locus, seven inbred rat strains were hyperimmunized with GASV and the resultant anti-SACHO antibody response quantified. The low response of all seven inbred strains suggests the non-association of immune response genes to SACHO with the major rat histocompatibility locus, Ag-B. This result supports similar observations in the mouse in which two inbred strains of the same histocompatibility locus demonstrated both a high and low anti-SACHO response (Braun, Kindred and Jacobson, 1972). It should be stressed, however, that classification of all seven inbred rat strains as low-responders to SACHO is a conclusion drawn with reference to the specific immunization schedule used. Further studies are in progress to assess the effect of additional immunization on the antibody response.

The development of the SACHO:anti-SACHO system in rats, whereby high levels of specific antibodies with restricted heterogeneity may be induced, provides an opportunity to elicit anti-idiotypic antisera and subsequently examine the expression of idiotypic determinants associated with the humoral response to SACHO. Idiotypic determinants have provided a useful marker in investigating the structure and biosynthesis of antibodies. For example, idiotype has been useful in probing the onset, persistence and replacement of antibodies during and following immunization (Nisonoff, MacDonald, Hopper and Daugharty, 1970; MacDonald and Nisonoff, 1970; Oudin and Michel, 1969a). Similarly, evidence for the biosynthesis of IgM and IgG antibodies by a single clone of cells has largely relied on idiotypic analysis (Oudin and Michel, 1969b). Most important, however, idiotypic determinants provide a useful tool in elucidating the mechanism(s) for the generation of antibody diversity.

The humoral response to antigenic stimulation may encompass a range from polyclonal to monoclonal. As previously discussed, the antibody response is influenced by a number of factors. Undoubtedly one of the most important and most basic influences is the genetic makeup of the individual. It might be expected that individuals with a similar genetic composition should respond similarly to antigenic challenge. Observations on idiotype have confirmed this assumption (Briles and Krause, 1972, 1974; Eichmann and Kindt, 1971; Nisonoff, 1974). The inverse of this statement should likewise prove true. That is,

individuals with similar responses to antigenic challenge should have similar immune genotypes. Applying this reasoning to the existing theories for the generation of antibody diversity, the expression of a similar humoral response among different individuals should support a germ-line theory rather than a somatic mutation theory. In this respect, idiotypic determinants provide an effective means of analyzing the occurrence in a population of similar genes coding for variable region antibody determinants in response to antigenic stimulation.

The observations on cross-idiotypic specificity of rat anti-SACHO antibodies strongly favor a germ-line theory for the generation of antibody diversity. Specific characteristics of the cross-idiotypic response of rats to SACHO can be summarized as follows:

1. ID1 represents antigenic determinants associated with or part of the antibody combining site. Several lines of evidence indicate that the cross-idiotypic specificity observed for rat anti-SACHO antibodies is dependent upon site-associated determinants. Inhibition of the idiotype:anti-idiotypic reaction in agarose gel, by both N-acetyl-D-glucosamine, the immunodominant determinant of SACHO and D-glucosamine hydrochloride at concentrations as low as 0.05 M and 0.15 M respectively, clearly implicates the antigen-binding site or an area adjacent to the binding site in determining the specificity of ID1. Moreover, rhamnose hydrate or anhydrous D-glucose at a concentration of 0.3 M did not inhibit

the idiotypic:anti-idiotypic reaction. The reaction of anti-idiotypic antisera with Fab' fragments prepared by pepsin digestion of pooled rat anti-SACHO antibodies containing ID1 revealed single lines of precipitation which were identical with the single precipitin lines formed between the anti-idiotypic antisera and the undigested antibodies. In an attempt to further localize ID1, the reaction of the anti-idiotypic antisera against isolated heavy and light chains prepared from rat IgG anti-SACHO antibody containing ID1 (R23G) likewise demonstrated single lines of precipitation which were identical to the single precipitin lines formed between the anti-idiotypic antisera and the unreduced antibody (R23G). Employing RIA, however, a more extensive analysis of the reactivity of isolated heavy and light chains with a rabbit anti-idiotypic antiserum demonstrated that although a major portion of the idiotypic determinant(s) may be expressed on anti-SACHO light chains, clear heavy-light chain interaction is required for the complete expression of ID1. Moreover, the ability of isolated light chains prepared from R23G to completely inhibit the idiotypic:anti-idiotypic reaction, further demonstrates that the idiotypic determinant(s) expressed on the light chains is present on the same antibody molecules expressing ID1 through heavy-light chain interaction. The conceivable argument that the cross-specificity observed with ID1 might at least in part be due to λ light chain isotypic determinants was resolved by employing the interspecies cross-reactivity of a chicken anti-human λ light chain antiserum. None

of 15 ID1 positive antibody preparations had detectable levels of λ light chains. Additionally, ID1 positive antibodies isolated from three separate animals possessed κ light chains only.

2. ID1 is apparently associated with rat anti-SACHO antibodies only.

This characteristic is actually a corollary of the first. The removal of ID1 activity by immunoadsorption of rat anti-SACHO antisera on N-acetyl-D-glucosamine immunoadsorbents provides strong evidence that ID1 is associated only with anti-SACHO antibodies since antibodies other than those with anti-SACHO activity should be present in the rat antisera. Additionally, the inability to detect ID1 in rat antisera to poly-GA obtained from animals which subsequently expressed ID1 on anti-SACHO antibodies suggests the association of ID1 exclusively with anti-SACHO antibodies. Finally, the inability to detect ID1 by RIA in preimmunization sera from all animals thus far examined (100) demonstrates the importance of specific antigenic stimulation for the induction of ID1.

3. ID1 represents a stable gene product. The remarkable stability in expression of ID1 is represented by its occurrence in all anti-SACHO antisera obtained from Sprague-Dawley rats selectively inbred seven generations for a low response to SACHO. Therefore, even by selectively restricting the response to low levels of anti-SACHO, the expression of ID1 is not diminished. Moreover, the unusually high frequency of

occurrence of ID1 in an outbred Sprague-Dawley rat population further argues for the conservation of specific antibody variable region genes.

4. The occurrence of ID1 is not restricted to a high antibody response, nor to any single rat histocompatibility haplotype or major rat allotypic light chain determinant. Interstrain analysis of the occurrence of ID1 in rat anti-SACHO antisera obtained from animals representing six major rat histocompatibility haplotypes at the B locus and both major rat allotypic light chain determinants demonstrated a high incidence of ID1 in all nine strains. Moreover, ID1 was equally detectable in anti-SACHO antisera from two F_4 rat families segregated into high- and low-responders to SACHO.

5. ID1 is a species-specific idiootype. Analysis of anti-SACHO antisera obtained from chickens, goats, and rabbits and specifically purified anti-SACHO antibody from nurse sharks confirmed the lack of expression of ID1 on anti-SACHO antibodies produced by species other than the rat. It should be mentioned that in the case of the chicken antisera, concentrations of antibody as high as 60 times the amount necessary to demonstrate significant inhibition with rat anti-SACHO antibodies were used as inhibitors in the RIA.

6. ID1 is apparently associated with a unique heavy chain variable region subgroup. The amino-terminal heavy chain sequence analysis of three separate anti-SACHO antibody preparations containing ID1, demonstrated identical N-terminal heavy chain sequences for all three antibodies up to the first hypervariable region (30 amino acids). Although the sequence closely approximated the V_{HIII} subgroup of human heavy chains, identical amino acid substitutions in all three anti-SACHO antibodies at positions 3, 16, 21 and 28 provide evidence for the existence of a new heavy chain subgroup of rat immunoglobulins. Moreover the expression of this subgroup is dependent upon immunization with GASV. Isolated IgG heavy chains obtained from an animal during non-immunization did not contain the four specific amino acid substitutions previously observed for anti-SACHO IgG heavy chains isolated from the same animal.

With general reference to all of the above observations, and more specifically to the necessity of specific antigenic stimulation for the induction of ID1, it should be noted that although the observations on cross-idiotypic specificity of rat anti-SACHO antibodies favor a germ-line theory for rat antibody diversity to SACHO, the general assumption of only germ-line genes coding for antibody variability is not apparent from idiotypic analysis of other antigen-antibody systems (Hart, Pawlak and Nisonoff, 1973; Eichmann, 1974). Undoubtedly one of the most

important features of any idiotypic analysis in which cross-idiotypic specificity is observed, is the suppression of the cross-reactive idio- type and an analysis of the resultant antibody response following immunization. Such an analysis should include both a measurement of the antibody titer in suppressed animals and an examination of idio- typic determinants following cross-idiotypic suppression. Only after performing such an experiment can an unequivocal statement be made concerning the variability in response of animals to a specific antigen, and the dependence of antibody variability solely on germ-line genes.

Hart, Pawlak and Nisonoff (1973) observed that the suppression of a cross-specific idio- type associated with the immune response of mice to azophenylarsonate did not affect the subsequent antibody titer following immunization and each suppressed mouse expressed individually- specific idiotypic determinants on anti-azophenylarsonate antibodies. Similarly, Eichmann (1974) noted that the suppression of cross-specific idiotypic determinants on A/J mouse antibodies to SACHO did not affect the resultant anti-SACHO antibody levels. Moreover, the anti-SACHO antibodies of suppressed A/J mice had individually specific isoelectric spectra. These observations taken together demonstrate that although mice may respond similarly to both azophenylarsonate or SACHO, suggesting the expression of germ-line genes coding for the antibody responses, additional alternatives in antibody variability are available.

An important consideration to the final interpretation of cross-idiotypic suppression studies, is that although animals may indeed express additional variability beyond the diversity encoded by germ-line genes, similar or identical alternatives are initially favored in response to antigenic stimulation. Present explanations for the selective expression of one variable region gene over another are yet incomplete. However, the evolutionary selection of genes coding for the variable regions of antibodies may reflect their importance in survival to a species (Sher and Cohn, 1972a). In the specific case of antibodies to SACHO, this reasoning would certainly seem relevant (Dudding and Ayoub, 1968; Goldstein, Halpern and Robert, 1968).

As an alternative explanation for the expression of a common idiotypic on rat anti-SACHO antibodies, the following reasoning is proposed (M. B. Rittenberg, personal communication). Intravenous hyperimmunization with large doses of carbohydrate antigen, may induce selective tolerance of "all" cell clones which produce high affinity anti-SACHO antibody. The resultant antibody response would reflect the survival of the fittest clones, i.e., those producing antibody of lowest affinity for SACHO. ID1 would represent, therefore, the most common variable region providing the minimum structural complementarity required to bind with SACHO. Based on general antibody responses in which initial low affinity antibodies are replaced by antibodies of higher affinity, there must be many more clones producing antibody of

low affinity than clones responding with high affinity antibody to SACHO. Although thermodynamic data are not available for the rat anti-SACHO:SACHO interaction, an experimental approach to this question is currently in progress. This proposal is not in conflict with the observations of Hart, Pawlak and Nisonoff (1973) or Eichmann (1974) and would account for the association of a unique heavy chain variable region subgroup with ID1 positive rat anti-SACHO antibodies.

Substantial evidence exists of immunological cross-reactivity between structural glycoproteins extracted from human or bovine heart valves (glycoprotein B) and the group specific antigen of group A streptococci, SACHO (Goldstein, Halpern and Robert, 1968). However, the obvious question of cardiovascular pathogenicity of antibodies elicited to N-acetyl-D-glucosamine, the immunodominant determinant of SACHO, remains unanswered. Initial studies by Goldstein, Halpern and Robert (1967) revealed that immunization of rabbits with either a whole valve homogenate or glycoprotein B produced precipitating and hemagglutinating antibodies which could be partially absorbed by N-acetyl-D-glucosamine. A relevant observation was the finding that sera from rheumatic patients also contained antibodies that reacted with the glycoprotein material.

Several lines of evidence favor the participation of SACHO in the initiation of the chain of events leading to rheumatic heart disease. First, all group A streptococci possess the C polysaccharide which may

explain the lack of specificity among different types of group A streptococci to induce rheumatic heart disease. Second, the carbohydrate residues are resistant to degradation and are not readily disposed of by the host's tissues. In this respect, Dudding and Ayoub (1968) have noted the prolonged persistence of anti-SACHO antibodies in patients with rheumatic valvular disease.

In view of these findings, the systematic evaluation of the humoral response of rats to SACHO provides a model system for an examination of the relevancy of anti-SACHO antibodies to cardiac pathology. The feasibility of employing the rat as an experimental model is strengthened by a) the ease with which rat heart cells are cultured, b) cross-reactive antigens localized both to group A streptococci and to rat heart cells (Wagner and Wagner, 1973), and c) multiple genetic influences on the humoral response of rats to SACHO. In reference to the latter, it is of interest to note the postulated hereditary susceptibility of certain humans to rheumatic heart disease (Taranta, Metrakos and Uchida, 1959; Taranta, 1961).

SUMMARY AND CONCLUSIONS

Hyperimmunization of Sprague-Dawley rats with a heat-killed, pepsin-treated vaccine of group A Streptococcus, elicited both precipitating and nonprecipitating antibodies to the group-specific carbohydrate of the Streptococcus. The occurrence of nonprecipitating antibodies in the immune response of the rat appeared to be genetically influenced by selective inbreeding that causes a low-precipitin response. A number of rats responded to immunization by producing electrophoretically restricted antibody components. This restriction in light chain heterogeneity confirmed by polyacrylamide gel electrophoresis of isolated antibody is apparently genetically influenced and capable of recall after extended periods of nonimmunization.

Selective breeding of Sprague-Dawley rats immunized with GASV revealed genetic influences on the magnitude of the precipitin response to SACHO. After four brother-sister inbreedings, a family of low-precipitin responders was segregated from a family of high-precipitin responders. Precipitin analysis of all four generations of rats bred for low-precipitin responses revealed an earlier influence of inbreeding on the secondary as compared to the primary precipitin response. Prolonged immunization and/or variation in the dose of antigen failed to enhance the magnitude of the precipitin response of low-precipitin responders. Analysis of the humoral response of seven inbred rat strains,

representing six major rat histocompatibility haplotypes at the B locus, suggests the non-association of immune response genes to SACHO with the major rat histocompatibility locus.

Affinity-immunoadsorbent chromatography was employed for fractionation of rat anti-SACHO antibodies of restricted heterogeneity. Evaluation of several eluting reagents including sodium thiocyanate, urea, N-acetyl-D-glucosamine and acid-salt demonstrated the chaotropic ion thiocyanate to be most effective in separating anti-SACHO populations of individual affinity characteristics.

Specifically purified antibodies to SACHO from both related and unrelated Sprague-Dawley rats were found to possess cross-specific idiotypic determinants (ID1). Additionally, cross-idiotypic specificity was demonstrated for anti-SACHO antisera obtained from nine inbred rat strains. Undetectable in preimmune sera, intimately involved in the antibody combining site and apparently associated with a unique heavy chain variable region subgroup, the occurrence of this species-specific, cross-reacting idio type (ID1) was not associated with antibodies to poly-GA produced by animals which subsequently expressed ID1 on anti-SACHO antibodies. The observations on idiotypy favor the importance of germ-line genes coding for rat antibody variable region determinants in response to SACHO.

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