THE IMMUNE NETWORK: ENDOGENOUS AND EXOGENOUS INFLUENCES ON IDIOTYPE EXPRESSION

by

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STATEMENT OF PROBLEM

Just as biosynthetic pathways represent a network of integrally regulated biochemical reactions, the immune response is a network of integrally regulated immunological reactions. The purpose of the studies presented in this thesis is to analyze autologous immune regulatory networks and to define influences which alter in vivo immune responsiveness as directed by these networks. Awareness of immune regulatory networks and the identification of probes which allow for their specific manipulation provide a means for most effectively regulating an individual's immune repertoire.

One approach which can be used to analyze immune regulatory networks is to monitor the ability of the immune system to recognize and respond to its own idiotypes. Many investigators have used anti-idiotypic antibodies to specifically suppress or enhance the production of idiotypes in animals (Eichmann 1974, Nisonoff et al. 1977, Cosenza et al. 1977, Wuilmart et al. 1979). These findings are consistent with the network theory of immune regulation proposed by Jerne in 1974 which states that self-idiotypic recognition serves as one means by which an individual regulates humoral and cellular immune reactivity in vivo. It is important to point out, however, that very few studies support the in vivo existence of autologous, idiotype specific regulatory networks following challenge by a foreign antigen, and these studies only indirectly imply their existence (McKearn et al. 1974, Kluskens and Kohler 1974, Cosenza 1976, Kelsoe and Cerny 1979, Schrater et al. 1979, Rodkey 1980). Due to the multiplicity of different idiotypes generally

induced by immunization with a specific antigen and to the regulatory influences induced by cross-reactive idiotypes, it is easy to understand why autologous idiotype specific regulatory networks have been difficult to discern in vivo.

In this thesis Id-1, a rat anti-SACHO idiotypic probe, was used to extensively analyze the potential of antigen induced endogenous idiotype-specific immunity and the potential of exogenously administered idiotypic or anti-idiotypic antibodies to alter in vivo immune responsiveness.

Id-1 is a cross reactive idiotype associated with rat anti-Group A streptococcal carbohydrate (anti-SACHO) antibodies (Stankus and Leslie 1974a, 1976a). The identification of Id-1 was facilitated by the production of high concentrations of restricted anti-SACHO antibodies by some rats following immunization with Group A streptococcal vaccine (GASV) (Stankus and Leslie 1974b). The preparation of heterologous antibodies against Id-1 allowed us to monitor and manipulate the production of Id-1 specific antibodies produced by what appears to be a small family of closely related B cell clones.

Two approaches were taken to determine if antigen induced autologous Id-1 specific immune reactivity exerted significant regulatory influences on immune responsiveness in vivo. First, levels of Id-1 and anti-SACHO were monitored following immunization with GASV, and the kinetics of antibody production were examined for evidence of autologous idiotype specific regulatory influences. Second, to enhance the direct recognition of possible permanent (Id-1 specific effects) regulatory influences exerted by the idiotypic network, I examined Id-1

specific effects induced by a mature immmune system (maternal immune system) on an immature and developing immune system (the immune system of her progeny).

Since it is known that exogenously administered idiotypic and anti-idiotypic antibodies have the potential to alter immune responsiveness in vivo, it becomes highly probable that idiotypic reagents can be used clinically as effective immunological manipulators. For example, using animal models, passive administration of anti-idiotypic antibodies or the induction of idiotype specific immunity in vivo was shown to suppress the growth of B cell lymphomas (Lanier et al. 1980), leukemias (Stevenson et al. 1977), myelomas or plasmacytomas (Beaty et al. 1976, Abbas and Klaus 1977, Bridges 1978, Sakato et al. 1979, Lynch et al. 1981), and to lessen the severity of autoimmune diseases (Brown et al. 1979, Zanetti and Bigazzi 1981). If idiotypic and anti-idiotypic antibodies are to be used clinically, however, it is essential that one have a precise understanding of the possible effects which will be induced by their in vivo administration. This is particularly important since it is highly unlikely that any two individuals would maintain the same immunological state at the time of administration of a particular idiotypic or anti-idiotypic antibody, due to the dynamic nature of the immune system. A greater understanding of the advantages and the limitations of these reagents in vivo, along with the identification of mechanisms compensating for individual immunological differences can be obtained from studies in animal models. A series of studies designed for such purposes were performed using Id-1 and anti-Id-1 antibodies.

One final intent of the studies in this thesis is to acquire a greater understanding of the potential of idiotypic manipulations to totally redirect the immune network. For example, of the numerous times we immunized rats with different antigens, e.g. SRBC, Streptococcus pneumoniae and poly-glutamic acid-alanine, in our laboratory, never did we find Id-1 to be associated with an antibody having other than specificity for SACHO (Stankus and Leslie 1977). The question arises: Can one induce Id-1 to be associated with an antibody of another specificity by stimulating the Id-1 regulatory network prior to stimulation with the non-SACHO antigen? It was also observed that Id-1 was not associated with anti-SACHO antibodies found in any species other than the rat (Stankus and Leslie 1977). Using a similar approach to the above, can one induce Id-1 anti-SACHO antibodies in another species by first stimulating Id-1 specific clones before challenge with GASV? Such studies would serve to enhance an understanding of the hierarchy of idiotypic immune regulatory networks.

REVIEW OF LITERATURE

Idiotypy

Individual antigenic specificities of immunoglobulin molecules were first recognized by Slater et al. in 1955 and in 1966 termed idiotypes by Oudin (1966a, b). Idiotypes represent antigenic determinants on immunoglobulin molecules which are not present on antibodies to the same antigen from other individuals, nor on antibodies to different antigens from the same individual. Since idiotypes serve to identify a

population of antibody molecules having a particular specificity, it was believed that one could use idiotypic probes to assess an individual's immunological repertoire. This, in turn, could allow one to estimate the number of immunoglobulin structural genes represented in the genome. In more recent studies, however, it became evident that regulatory genes also played in important role in determining whether certain immunological structural genes would or could not be expressed (Cosenza et al. 1977, Sigal 1977, Bosma et al. 1978, Cancro et al. 1978, Wilker et al. 1979, Bona and Paul 1979, Gearhart and Cebra 1979, Yarmush et al. 1979). Thus, although the idiotypic repertoire expressed by an individual is immense, it still remains to be an inadequate representation of their immunological potential. The role of idiotypes in analyzing immune responsiveness, consequently shifted from the gene level to the level of regulation. The questions arose: a) what is the actual role of idiotype-anti-idiotype interactions in the regulation of immune responsiveness in vivo, and b) why is a particular idiotype produced by only certain individuals, although others may have the genetic potential to produce the same idiotype?

Regarding the first question, there is considerable evidence supporting the potential for idiotype-anti-idiotype interactions to regulate immune responsiveness (for review see Rodkey 1980), as would have been predicted from the idiotypic network theory of immune regulation (Jerne 1974). The significance of idiotypic interactions during a normal immune response, however, remains to be proven. Due to the complexity of idiotype-anti-idiotype interactions, it is not difficult to understand why alterations in the concentration of a specific

antibody population (one of a broad spectrum of antibodies induced by a specific antigen) might be extremely difficult to detect. Such complexity, logically, would hinder the recognition of the involvement of idiotypic interactions in immune regulation. This problem, however, is alleviated by using an experimental system that limits the heterogeneity of idiotype expression, and, in turn, simplifies the recognition of idiotype-anti-idiotype regulatory influences.

In reference to the second question, idiotypic similarities amongst antibodies produced by different individuals have extended the definition of idiotypes beyond that originally proposed by Oudin. Although examples of such idiotypes in humans and animals show only partial identity (cross-reactive or public idiotypes) (Williams et al. 1969, Kunkel et al. 1972, Briles and Krause 1974, Wolfe and Claflin 1980), some rabbit families and some inbred mouse strains show very similar idiotypic specificities on antibodies directed against the same antigen (Eichmann and Kindt 1971, Kuettner et al. 1972, Eichmann 1975, Claflin and Cubberley 1978).

In 1977, Urbain et al. and Cazenave, from separate laboratories, showed that one could "turn on" the production of a specific idiotype in rabbits normally lacking its expression by stimulating the idiotypic system, prior to challenge with antigen. Other investigators have also shown that the administration of anti-idiotypic antibodies prior to antigenic challenge could enhance the animal's subsequent ability to produce idiotype (Trenkner and Riblet 1975, Hiernauz et al. 1981). A possible explanation for the natural absence of the production of a

particular idiotype was provided by Bona and Paul (1979), who attributed the lack of expression of the MOPC 460 idiotype in BALB/c mice to the natural presence of an idiotype-specific suppressor cell. In vitro depletion of this regulatory T cell population before culture significantly increased the number of plaques secreting anti-TNP antibodies bearing MOPC 460 idiotype. These studies provide evidence that the immune repertoire expressed by an individual is actually a reflection of genetic regulatory tendencies and past immunological experiences, and that this expressed repertoire has the potential to be altered if the appropriate external manipulations are performed.

Idiotype Specific Immune Regulation

It is well documented that exogenous anti-idiotypic antibodies can have suppressive effects on idiotype production (Hart et al. 1972, Eichmann 1974, Strayer et al. 1975), and under specified conditions may have an enhancing effect on antibody production and the expression of an idiotype (Eichmann and Rajewsky 1975, Trenkner and Riblet 1975). Antigen induced autologous anti-idiotypic antibodies have been detected in some animals, but their induction is difficult to recognize and generally requires repeated immunization with the antigen (Kluskens and Kohler 1974, McKearn and Fitch 1975). Both isologous and autologous anti-idiotypes have been prepared by immunization with chemically modified or polymerized antibody molecules possessing the idiotype (Rodkey 1974, Sakato and Eisen 1975, Kohler et al. 1978). Cosenza et al. (1977) observed that the induction of auto-anti-idiotypic antibodies against T15 anti-phosphorylcholine (PC) idiotype occurred more readily in mice

neonatally suppressed for this idiotype than in non-suppressed mice. Also, Strayer and Kohler (1976) detected auto-anti-idiotypic antibodies against the PC receptor in normal neonatal BALB/c mice, and Goidl et al. (1979) detected auto-anti-idiotypic antibodies during the normal response of mice to TNP-Ficoll.

Evidence that idiotype-anti-idiotype interactions might be involved during a normal immune response originated from studies on animals immunized over prolonged periods. When antibody levels were monitored during the course of immunization, it was noted that the production of a specific antibody followed a cyclical pattern, often complementary to the production of a specific anti-idiotype (Kluskens and Kohler 1974, McKearn et al. 1974, Cosenza 1976, Urbain 1976, Kelsoe and Cerney 1979). If this cyclical pattern were analyzed in relation to the expression of various idiotypes, a continual alteration or shifting of the idiotype was observed, as if each specific antibody clone had a finite existence after which it was replaced by another population (MacDonald and Nisonoff 1970). Similarly, Rowley et al. (1976) and Augustin and Cosenza (1976) while noting complementary levels of idiotype and anti-idiotypic antibodies in the PC T15 idiotype system of mice, recognized the "priority of the first response". That is, if mice are producing high levels of the T15 idiotype at the time of challenge with PC, these animals produce high levels of the T15 positive anti-PC antibodies. Conversely, if mice are producing high levels of anti-T15 just before PC immunization, their anti-PC antibodies lack the T15 idiotype.

The recognition of idiotypic determinants on T and B cells, identical to idiotypic determinants on antibody molecules, further supports the involvement of idiotypic interactions in the "triggering" of specific lymphocyte populations (Cosenza and Kohler 1972, Granato et al. 1974, Binz and Wigzell 1975 a, b, Strayer et al. 1975, Geczy et al. 1976). In this regard, both idiotypic and anti-idiotypic determinants have been found to serve as functional receptors on T-helper cells (Eichmann and Rajewsky 1975, Janeway et al. 1975, Black et al. 1976, Cosenza et al. 1977, Julius et al. 1977, Woodland and Cantor 1978, Bottomly et al. 1978, Hetzelberger and Eichmann 1978), T-suppressor cells (Owen et al. 1977, Bona and Paul 1979, Kim 1979, Sy et al. 1980, Hirai and Nisonoff 1980), T-effector cells (Ramsier 1973, Binz and Wigzell 1975a, 1978), and B cells (Trenkner and Riblet 1975, Binz and Wigzell 1978, Brown and Rodkey 1979, Wilker et al. 1979, Cosenza 1979).

When cell surface idiotype expression was examined in relation to its co-expression with other antigenic determinants on immunoglobulin molecules, it was found by some investigators that idiotypic determinants on T-cells closely resemble those found on heavy (H) chains but not light (L) chains (Krawinkel et al. 1977, Cazenave et al. 1977). Others, however, were unable to identify any immunoglobulin H- or L-chain determinants in association with T-cell idiotypic determinants (Binz and Wigzell 1976). In agreement with the association of idiotypes with immunoglobulin H-chains on T cells is the increasing evidence linking the expression of inherited idiotypes to specific H-chain allotypes (Eichmann 1973, Krause 1974, Weigart and Potter 1977, Cancro et al. 1978). A lack of recognizable idiotypic linkage to L-chain markers

may be due to the lack of V_L polymorphism as has been recently proposed by Laskin et al. (1977) or to the difficulty in identifying L-chain allotypic markers (Sogn et al. 1976). Idiotypic determinants on B cells are generally accepted as being located on 7S immunoglobulin molecules, and surface IgD and IgM bear identical idiotypes (Fu et al. 1975).

Maternal Influences on Progeny immune Responsiveness

Permanent alterations in idiotype expression often occur more readily when naive cells, rather than mature cells are exposed to specific anti-idiotypic antisera, e.g., anti-idiotypic antibodies, usually induce permanent idiotypic suppression when injected into neonatal animals, but not when injected into adults (Kohler 1975, Strayer et al. 1975, Augustin and Cosenza 1976, Bona et al. 1979). Similarly, Pierce and Klinman (1977) using a splenic focusing technique, identified an antigen specific regulatory mechanism, believed to be idiotype related, which specifically limited the responsiveness of primary cells (unexposed to hapten prior to cell transfer) but did not limit the response of secondary cells which had seen hapten in vivo.

In relation to this, considerable evidence has been presented emphasizing the important influence the maternal immune system can have upon immune responsiveness of her progeny (Dray 1962, Jacobson and Herzenberg 1972, Solomon et al. 1972, Kindred and Roelants 1974, Auerbach and Clark 1975, Stern 1976, Halsey and Benjamin 1976, Gill et al. 1977a, Beer et al. 1977, Minami et al. 1977, Sasaki et al. 1977).

It is therefore apparent that some component(s) within the maternal system, whose presence may be transiently induced by an antigenic stimulus, has the potential to be transferred to developing young and subsequently cause an alteration of immunological responsiveness. Several studies have been directed at identifying the specific maternal component responsible for inducing this alteration, but, as yet, its actual identity remains controversial. While some investigators have implicated maternally derived antibody to a particular antigen [via a "feedback" mechanism (Uhr and Bauman 1961)] inducing altered immune reactivity (Dray 1962, Solomon et al. 1972, Minami et al. 1977, Sasaki et al. 1977) others have attributed this alteration to transfer of maternal lymphocytes (Field and Caspary 1971, Stern 1976, Beer et al. 1977). Soluble T cell factors (Field and Caspary 1971, Stern 1976, Barnetson et al. 1976), antigen-antibody complexes (Auerbach and Clark 1975) or maternally derived antigen (Kindred and Roelants 1974, Halsey and Benjamin 1976, Gill et al. 1977a) have also been implicated.

In addition to the controversy concerning what maternal component was responsible for the altered immune responsiveness of progeny, there are reports of varying effects induced in offspring by these maternal factors. Some investigators have observed that offspring of females immunized with a particular antigen have enhanced immune responsiveness against that antigen (Field and Caspary 1971, Stern 1976, Minami et al. 1977, Susaki et al. 1977, Gill et al. 1977b), while others have observed specific immune suppression (Auerbach and Clark 1975, Gill 1975, Halsey and Benjamin 1976) or qualitative rather than

quantitative changes in progeny immune responsiveness (Kindred and Roelants 1974).

Regardless of the type of regulatory mechanism or the exact influence exerted, it is highly probable that maternally induced regulatory influences on progeny immune responsiveness could serve as a means of detecting subtle immune regulatory mechanisms, such as idiotype specific regulation. The maternofetal test system offers this advantage because it allows a natural intermeshing of immunologically mature, maternal immune reactivity with immature and more permanently susceptible neonatal immune reactive cells (Raff et al. 1975, Metcalf and Klinman 1976, Cambier et al. 1976, Fidler 1979, Teale and Mandel 1980). In other words, females immunized with antigen to induce the synthesis of antibodies with specific idiotypes and, in turn, anti-idiotypes, could transfer these antibodies or immune cells to their offspring where they may permanently affect the clonal repertoire. The induction of such influences on neonatal animals would allow the effects of idiotype reactive cells or antibodies, which remain undetectable within the mother, to be recognized.

Evidence that idiotype specific immune reactivity induced in females can affect progeny idiotype expression was presented by Weiler et al. (1977) and Cosenza et al. (1977). In these studies, progeny of either idiotype immune or idiotype suppressed females were suppressed for idiotype and total antibody production. Binz and Wigzell (1976) also observed that female Lewis rats that produced anti-idiotypic antibodies against Lewis anti-DA rat T-cell receptors, apparently

transferred to progeny a factor responsible for the specific reduction in mixed lymphocyte culture reactivity against DA alloantigens. These studies emphasize that maternal synthesis and transfer of anti-idiotypic antibodies and/or cells suppress the developing offspring's immune responsiveness and lend support to the possibility that maternal idiotypes and anti-idiotypes produced during a normal immune response against an antigen can also alter the immune potential of progeny.

It is well recognized in humans that maternal antibodies and lymphocytes are transplacentally transferred and can affect the neonate (Loke 1978). However, these maternal influences have generally been considered transient, lasting only until the maternal factor or cell was eliminated. Studies in animal systems (Dray 1962, Kindred and Roelants 1974, Ono et al. 1974, Auerbach and Clark 1975, Stern 1976, Minami et al. 1977, Weiler et al. 1977, Beer et al. 1977, Loor and Kelus 1978, Jarrett and Hall 1979) and some studies in humans (Peer 1958, Field and Caspary 1971, Barnetson et al. 1976, Evans et al. 1976) provide evidence that the maternal immune system can permanently affect immune responsiveness of offspring. An important outcome of defining and understanding mechanisms of materno-fetal interactions concerns its direct applicability to studies of disease etiologies and immunological engineering (e.g. if antigen induced idiotype specific influences of maternal origin result in the selective stimulation of specific antibody producing clones within the developing neonate, this not only has the potential for increasing the amount of antibody synthesized by an individual against a particular infectious agent, but

also maximizing the immune reactivity of the individual by selectively stimulating the clone of cells known to produce the most efficient immune response). Conversely, similar maternally derived influences, rather than enhancing, may specifically debilitate immune responsiveness of the child. Such a mechanism may serve to explain the acquisition by certain individuals of hypersensitivities or autoimmunities which appear to be inherited but in an unpredictable pattern.

The Clinical Use of Idiotypic Manipulations

As referred to above, anti-idiotypic antibodies, and idiotype and anti-idiotype bearing lymphocytes have all proven to be successful modalities for altering idiotype production. With these observations, the potential for using idiotypic and anti-idiotypic antibodies as specific immunoregulatory probes in clinical situations also becomes increasingly more plausible. In relation to pathological states, passive administration of anti-idiotypic antibodies or the induction of idiotype specific immunity in vivo was shown: a) to suppress the growth of murine B cell lymphomas (Lanier et al. 1980), murine myelomas or plasmacytomas (Beaty et al. 1976, Abbas and Klaus 1977, Bridges 1978, Sakato et al. 1979, Lynch et al. 1981), and guinea pig leukemias (Stevenson 1977); b) to inhibit or lessen the severity of autoimmune diseases such as tubulointerstitial nephritis in guinea pigs (Brown 1979) and autoimmune thyroiditis in rats (Zanetti 1981); and c) to specifically suppress transplantation immunity allowing prolonged graft survival (Binz and Wigzell 1976).

The primary advantage of idiotypic manipulations over other exogenous immunological manipulations is their precision. The administration of idiotypic or anti-idiotypic antibodies allows one to selectively suppress or enhance specific immune reactive clones, generally without affecting overall immune responsiveness. One also has the potential to alter the effective spectrum of a particular anti-idiotypic reagent by: a) using preparative techniques to increase the restriction of the idiotypic immunogen; b) altering the recognizable form of the immunogen, (i.e., injecting idiotype-antigen complexes); or c) varying the species in which the anti-idiotypic reagent is prepared (Rodkey 1976, Fritz and Desjardins 1981). Anti-idiotypic antibodies prepared against both homogeneous and heterogeneous idiotypes have proven to be successful immunoregulatory reagents, and cross-reactivities amongst idiotypes have extended the immunoregulatory potential of some anti-idiotypes to different individuals of the same species (Kunkel et al. 1972, Briles and Krause 1974) and even to those of different species (Ju et al. 1978).

The limitation of using idiotypic reagents clinically is the inability to reliably predict their immunological effect following their in vivo administration. This is particularly important in lieu of the fact that the administration of anti-idiotypic antibodies has been shown to induce either enhanced or suppressed idiotype production in different idiotypic systems. Thus, before these probes can be used in the treatment of human diseases, one must have an increased understanding of the mechanism by which these dichotomous results are induced.

The Id-1 Idiotype

For all studies presented in this thesis the Id-1 idiotypic probe was used. Id-1 is a complex idiotype present on rat antibodies directed against the carbohydrate moiety of Group A streptococcal cell walls. Originally Id-1 was obtained from a single Sprague Dawley (SD) rat, which, after hyperimmunization with GASV, produced anti-SACHO antibodies of highly restricted heterogeneity (1974a). As previously reported by Stankus and Leslie (1974a, 1976a, 1977), Id-1 requires both heavy and light chains for most complete expression and, as confirmed by hapten (N-acetylglucosamine) inhibition studies, is associated with the antibody combining site (Stankus and Leslie 1974a).

Intra- and interspecies analysis of the distribution of Id-1 indicated that this was a crossreactive idiotype present on the anti-SACHO antibody molecules of different strains of rats, but not on anti-SACHO antibodies of rabbits, chickens, sharks or goats (Stankus and Leslie 1976a, 1977). The genetic inheritance of Id-1 structural genes in rats was highly suggestive of a germline origin. Phenotypically, however, Id-1 was not inherited in strict Mendelian fashion (Stankus and Leslie 1975, 1976a). This implied that its mechanism of expression was a multigenic phenomenon, possibly under the influence of multiple regulatory mechanisms.

IgG anti-SACHO antibodies are predominantely, but not exclusively, of the IgG_{2c} subclass, as is Id-1 (Leslie 1979). The association between Id-1 and IgG_{2c} is not absolute, however, since IgG_{2c}-positive-Id-1-negative anti-SACHO antibodies are produced (Leslie 1979). In

addition a correlation between Id-1 expression and the IgG to IgM ratio of anti-SACHO antibodies was observed. A comparison of antibodies produced by SD rats selectively bred for high levels of precipitating anti-SACHO antibody (HPR) to those selectively bred for low levels of precipitating antibodies (LPR) revealed that LPR rats, although lacking the high level of precipitating anti-SACHO antibody present in HPR, did produce considerable non-precipitating anti-SACHO antibody. These antibodies had a lower incidence of Id-1, coincident with lower IgG to IgM ratio, than anti-SACHO antibodies produced by HPR rats (Stankus and Leslie 1974b).

Antigens generally elicit a heterogeneous population of antibody molecules bearing many different idiotypes yet maintaining specificity for the same antigen. GASV, as an antigen, offers the distinct advantage of being able to induce a heterogeneous or a highly restricted anti-SACHO antibody response depending upon the individual animal. This makes SACHO a versatile antigen for analyzing immune regulatory influences. Id-1 is associated with from 0 to >90% of the anti-SACHO molecules produced by an individual animal.

A heterologous anti-idiotypic reagent prepared against the $F(ab')_2$ fragment of Id-1 positive, specifically purified anti-SACHO antibody molecules was used to identify and quantify Id-1 in the studies in this thesis. Since this was not the same anti-Id-1 preparation used originally by Stankus and Leslie, the thesis begins with an extensive analysis of this new anti-Id-1 antibody preparation.

MATERIALS AND METHODS

Bacterial Strains

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

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

Phosphate buffered saline (PBS). A 0.01 M isotonic phosphate buffer was prepared by dissolving 1.8 g $Na_2HPO_4 \cdot 7H_2O$, 0.45 g $NgH_2PO_4 \cdot H_2O$ and 8.5 g NaCl in 1 liter of distilled water. The final pH was 7.2.

Tris-buffered saline. A 0.01 M "Tris 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.

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.

Immunoelectrophoresis buffer. This buffer was prepared by adding 5.4 g sodium barbital, 2.3 g sodium acetate, 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.

Experimental Animals

Rats. Male and female, 250-350 g outbred Sprague Dawley (S/D) rats were originally supplied by Dr. K.F. Burns of Tulane University Medical School. These rats were selectively brother-sister inbred in our laboratory for more than eight years based upon the production of high concentrations of precipitating anti-SACHO antibodies and are refered to as the high precipitin responder (HPR) rat strain.

Inbred Fischer 344 (F344) rats, a low responding strain to SACHO, were purchased from Simonsen Laboratories, Gilroy, California. Inbred August and Wistar Furth rats were obtained through the courtesy of Clarence Reeder of the Veterinary Resources Branch of NIH, Bethesda, Md. All three strains were further bred within our laboratory.

Rats were maintained on OSU rat chow(Corvallis, OR) and allowed continual access to water. Routinely, 0.09% aureomycin was added to the water to maintain the health of the animals.

Chickens. S/C chickens, B2/B2 homozygous, were obtained from Hy-line International Dallas Center, Iowa. The chickens were maintained on Albers Triple Duty Chick Feed (Carnation, Los Angeles, CA) and allowed continual access to water. To decrease the lipid content of blood, chickens were fasted 24 hours prior to bleeding.

Antigens

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). Twenty liters of an 18 hr Todd-Hewitt broth culture of the streptococci were heat-killed at 60 C for 90 min and formalin treated (0.03%). 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 (2000 g for 30 min) and the cells washed several times with sterile saline. The streptococcal pellet (15-20 g wet weight) was suspended in 500 ml of sterile saline, adjusted to pH 2 and containing 1 g 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.

GASV-DNP. Cyanogen bromide (CNBr: Eastman, Rochester, N.Y.) activated GASV was used to couple GASV to ε-DNP-lysine (DNP: Sigma, St. Louis, Mo.). Briefly 100 mg (dry weight) GASV was suspended in 5 ml of water and adjusted to pH 11 with NaOH. Upon addition of 10 ml CNBr (1 g/10 ml water) the pH was quickly adjusted and maintained at ∿10.5-11.5 with 1 N NaOH until the pH stabilized (∿15 minutes). The mixture was centrifuged in the cold at 3,000 rpm for 20 minutes, washed with 50 ml of 0.5 M $NaHCO_3$ (pH 9), resuspended in 10 ml $NaHCO_3$ containing 20 mg DNP and stirred at 4 C overnight. The GASV-DNP was then washed twice with Tris buffer.

Pneumococci. Streptococcus pneumoniae Type S8, suspended in 0.15 M NaCl solution with 0.1% formalin, was a kind gift of Dr. L.W. Clem, University of Mississippi, Jackson, MS. The cells were diluted to 5 x 10^9 cells/ml with saline and injected iv using the GASV "standard immunization protocol".

Sheep red blood cells (SRBC). A 10% (v/v) packed SRBC suspension was made on a weekly basis by washing SRBC (Prepared Media, Portland, OR) twice with saline (centrifuging at 2600 rpm for 40 minutes) and resuspending the cells in saline. The 10% solution was injected iv using the "standard immunization protocol" stated for GASV.

Animal Immunization Protocols and Injections

Immunization with GASV. For primary immunization, beginning at 3 months of age, the rats received three weekly intravenous (iv) or intraperitoneal (ip) injections of 1 mg of GASV, on consecutive days, for two or three weeks (Standard Immunization Protocol). Some rats were periodically rechallenged with GASV as indicated in the individual study. Chickens were immunized with GASV iv at 3 months of age. They were given 1 mg of GASV in week 1 and three injections of 1 mg of GASV on consecutive days during week 2. The chickens were further rechallenged with GASV as indicated in the study.

Immunization with GASV-DNP. Normal or GASV primed rats were challenged with GASV-DNP according to the ascitic fluid induction protocol (below). A total of 6 injections of 1 mg of DNP-GASV were given over an 8 week period.

Exposure of neonatal and adult rats to Id-1 or anti-Id-1. In several studies neonatal or adult rats were injected with anti-Id-1, Id-1 or immunized with Id-1. The details of the injections for each of these studies are indicated in the individual papers which follow.

Spleen cell transfers. Spleens from Id-1 suppressed females were minced, cell suspensions made and washed in Minimum Essential Medium (MEM: Grand Island Biological Co., Grant Island, NY). The cells were resuspended for 5 minutes at room temperature in 9 parts 0.83% ammonium chloride:1 part 0.2 M Tris buffer (pH 7.4) to lyse red blood cells. Leukocytes were then washed 2 times in MEM, resuspended in MEM to a concentration of $1x10^7$ per 50 µl and 50 µl injected ip into newborn rats.

Induction of Ascites

Production of large volumes of ascitic fluid (ASF) can be induced in rats by repeated ip injections of 1 ml of an emulsion consisting of a 9:1 ratio of Freund's complete adjuvant (FCA: Difco, Detroit, MI) to antigen or saline. Antigen primed or unprimed rats of different strains were injected initially with a 9:1ratio of Freund's incomplete adjuvant (FIA: Difco, Detroit, MI) to antigen. The purpose of the initial inoculation in FIA was to minimize ascites accumulation while increasing serum antibody levels (Tung et al. 1976). Two weeks after the initial inoculation of emulsion, rats were injected ip with an emulsion consisting of a 9:1 ratio of FCA to antigen. These injections were repeated weekly for 4 to 8 weeks. As ASF accumulated it was collected with an 18-gauge, 1.5 inch hypodermic needle, with or without a syringe.

For some experiments heparin (Panheparin, Abbot Laboratories, North Chicago, Il.) was mixed with ascitic fluid (1 unit/ml) to minimize clotting. Samples were centrifuged at 2000 rpm for 20 minutes and assayed for protein and antibody content. Antibodies were concentrated from large pools of ASF by precipitation with half-saturated ammonium sulfate [$(NH_4)_2SO_4$:Mallinckrodt, St. Louis, MO] .

Analysis of Immunoglobulins and the Preparation of IgG and IgG Fragments

Double diffusion in agarose gel (Ouchterlony). Ouchterlony analysis was performed in 50 by 9 mm petri dishes (Falcon, Oxnard, CA) coated with 1% (w/v) Seakem agarose in immunodiffusion buffer. When required to visibly enhance precipitation, 2% (w/v) polyethylene glycol 4000 (Baker, Phillipsberg, N.J.) was added to the agarose (Lundkvist and Ceska 1972).

Immunoelectrophoresis (IE). Immunoelectrophoretic analysis was performed in IE agar prepared by dissolving 10 g of Seakem agarose 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.

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. To quantify the relative amount of protein in each band, the stained cellulose acetate membranes were scanned using a Quick Scan densitometer (Helena Laboratories, Beaumont, TX).

Gel filtration. To separate proteins based on their molecular weight, protein solutions (<5 ml volume), adjusted with a 50% sucrose solution to be 5% sucrose, were applied to an upward flow Sephadex-G-200, 100 x 2.5 cm column (Pharmacia, Uppsala, Sweden). An equal volume of a 10% sucrose solution followed the protein mixture.

Sephadex columns were calibrated with 10 mg each of normal rabbit IgG (150,000 daltons), human transferrin (90,000 daltons) ovalbumin (43,000 daltons), and a 0.2% solution of blue dextran (∿1,000,000 daltons).

Preparation of rat IgG. Rat IgG was isolated from pooled whole rat serum by diethylamonoethyl-cellulose ion exchange chromatography (DEAE). The DEAE column (1 ml DEAE per 10 mg protein) was equilibrated with 0.005 M phosphate buffer pH 7.5. Rat serum, previously dialyzed against 0.005 M phosphate buffer, was applied to the column, and sequentially eluted with 0.005 M, 0.01 M, 0.05 M phosphate buffer (pH 7.5) and 0.05 M phosphate buffer with 0.05 M NaCl added (pH 7.5). The flow rate of the column was 1 drop per second. The 0.005 M or 0.01 M eluates contained predominantly the IgG_{2a} and IgG_{2b} subclasses, while all IgG subclasses were apparent in the 0.05 M phosphate eluate and the 0.05 M phosphate with 0.05 M NaCl eluate. This latter eluate also contained IgA.

Preparation of 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.

Preparation of rat Fab' and $F(ab')_2$ fragments. The procedure for the preparation of these fragments is described in "Characterization of Id-1" section.

Preparation and Purification of Antibodies

Preparation of affinity immunoadsorbent columns. To activate Sepharose (Pharmacia, Uppsala, Sweden), 1 g of CNBr (dissolved in 10 ml of distilled water) was added to 10 ml of packed, washed Sepharose 4-B (resuspended in an equal volume of distilled water). The mixture was immediately brought to and maintained at pH 11 with 4 N NaOH until the pH stabilized (~10 minutes). When the pH became constant, a large volume of distilled water was added, and the mixture was transferred to a sintered glass filter where washing with cold distilled water were continued. This was followed by washing with 20 volumes of 0.5 M sodium bicarbonate ($NaHCO_3$) pH 9. The activated Sepharose was then

transferred to a container where an equal volume of 0.5 M $NaHCO_3$ and the protein solution to be coupled (2.8 mg protein/ml Sepharose) were added. This mixture was mixed gently overnight on a rotator. To remove unbound protein, the immunoadsorbent was transferred to an appropriate sized column and washed with Tris buffer followed by 3 M sodium thiocyanate (NaSCN), then rewashed with Tris buffer. To prepare the N-acetyl glucosamine immunoadsorbent, the above procedure was used with the following alterations: 1) 5 g of CNBr was added to 100 ml packed Sepharose; 2) A 0.1 M Na_2CO_3 (pH 9) buffer was used for washes; and 3) 1.25 mg of p-aminophenyl-β-N-acetylglucosamine per ml of Sepharose was added in place of the protein (Rotta et al. 1971 as modified by Stankus and Leslie 1976a).

Affinity purification of antiserum. To purify antisera, sera were centrifuged to remove particulate matter and applied to the desired immunoadsorbent column. Unbound protein (effluent) was removed by washing with Tris buffer. Specifically purified antibodies were obtained by eluting immunoadsorbent columns with either 1, 2 or 3 M NaSCN. These eluates were dialyzed against Tris buffer and concentrated to the original serum volume.

Specifically purified anti-SACHO antibodies were prepared by sequential elutions off an N-acetylglucosamine immunoadsorbent column in the following stages: 1) non-immunospecific serum components were eluted with Tris buffer at 4 C (the temperature at which the column is initially run); 2) low-affinity specific components were removed by transfer of the column to room temperature and elution with Tris buffer at 20 C; 3) hapten-specific anti-SACHO antibodies were eluted with

15 mM N-acetylglucosamine; and 4) high affinity antibodies were eluted with either 1, 2 or 3 M NaSCN, as referred to above.

Preparation of anti-Id-1. The preparation of rabbit anti-Id-1 is described in detail in "Characterization of Id-1" section.

Anti-rat class and subclass antisera. Anti-rat IgM, IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c} and IgA were a kind gift of Dr. G.A. Leslie and prepared as described previously (Leslie 1979). Unlike earlier studies, however, the antisera used in this thesis were made against specific classes or subclasses of rat immunoglobulins isolated from normal rat serum. Previously, rat myeloma proteins served as immunogens. Anti-rat light chain and anti-normal rat serum were also a gift of Dr. G.A. Leslie.

Quantification of Antibodies

Purification of streptococcal A carbohydrate (SACHO). Carbohydrate was isolated from Group A streptococcal cells by a hot nitrous acid procedure (Kholy et al. 1974). Briefly, 100 mg (dry weight) of GASV was suspended in 5 ml of saline. To this was added 10 ml of 4 M sodium nitrite and 5 ml glacial acetic acid. The mixture was capped and allowed to sit for 30 minutes at 20 C. The first extraction product (supernatant) was obtained by centrifuging the mixture at 2000 rpm (4 C) for 15 minutes. To obtain extraction product 2, the above procedure was repeated on the remaining pellet. Both extraction products 1 and 2 were adjusted to pH 7.5 with 4 M NaOH and dialyzed against distilled water. After a minimum of 24 hr of dialysis the extraction products were centrifuged at 15,000 rpm for 30 minutes. 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.

Radiolabeling with iodine-125. The procedure used for the trace iodination of protein is that previously described by McConahey and Dixon (1966). Briefly, 0.5 mCi of ^{125}I was added to 0.5 mg of protein, diluted in 0.5 ml of 0.05 M phosphate buffer (pH 7). The mixture was maintained in an ice bath. Immediately 0.2 ml of cold freshly prepared chloramine T (400 µg/ml in distilled water) was added and the mixture was stirred for ∿4 minutes. To stop the reaction, 0.2 ml of cold sodium metabisulfite ($Na_2S_2O_5$) (400 µg/ml) was added.

Unbound iodine was removed from the mixture either by dialysis against 0.05 M phosphate buffer or by passage over a Sephadex G-25 column (Pharmacia, Uppsala, Sweden).

Detection and quantitation of anti-SACHO by reverse radial immunodiffusion (RRID). RRID plates were prepared by mixing either 1-2 µg of SACHO per 1 ml of 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 microscopic 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). In order to quantify the concentration of anti-SACHO antibody in chicken sera an additional 8 g of NaCl was added to 100 ml of the agarose solution.

Quantitation of anti-DNP antibodies. Anti-DNP antibodies were quantified by RRID in which 10 µg/ml of DNP-BSA (prepared as described by Garvey et al. 1977) was incorporated into agarose gel employing low salt buffer.

Quantitative precipitin analysis. Quantitative precipitin tests were performed in Tris buffer with 0.011 M EDTA by using 0.1 ml of a dilution of antiserum (either anti-SACHO or anti-DNP) and a range of concentrations of the appropriate antigen (SACHO or DNP-BSA) from 0 to 90 µg 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 (OD) determined at 280 nm in a Beckman Model 24 spectrophotometer (Fullerton, Ca.). The total protein in the precipitates was determined by using E 1% 280 nm; 1 cm equals 14.6 for rat immunoglobulin (Binaghi and Benacerraf 1964). The absorption of DNP was corrected for by determining the 280:360 nm OD ratio of DNP-BSA, multiplying this ratio by the OD of the solubilized precipitate at 360 nm, and subtracting this from the OD of the precipitate at 280 nm.

Quantitation of Id-1. Id-1 radioimmunoassay (RIA) is described in detail in the section "Characterization of Id-1".

Quantitation of anti-SACHO antibodies. Total anti-SACHO antibodies were assayed by radioimmunoassay (RIA) using a modified Farr technique (Bernstein et al. 1975). Purified SACHO, prepared as previously described (Stankus and Leslie 1975) was reacted with cyanogen bromide (CNBr) and coupled to tyramine. Specifically, 2.5 mg (dry weight of SACHO was suspended in 5 ml of distilled water. Upon addition of 5 mg CNBr (Eastman, Rochester, NY) dissolved in 0.25 ml of distilled water, the mixture was quickly adjusted and maintained at pH 11 with 1 N sodium hydroxide until the pH stabilized. Tyramine (Sigma, St. Louis, Mo.), 10 mg dissolved in 2 ml of 0.5 N sodium bicarbonate buffer (pH 8.5), was subsequently added and the pH of the mixture was readjusted to 8.5. After overnight stirring at 4 C the preparation was dialyzed against 0.1 M phosphate buffer, pH 7.0, to remove unbound tyramine. Tyraminated SACHO was radiolabeled with 2.5 mCi of ^{125}I (New England Nuclear, Boston, Ma.) using a chloramine T method (McConahey and Dixon 1966). To assay for anti-SACHO antibodies, dilutions of unknown serum samples were incubated with ^{125}I-SACHO at 37 C for 1 hr, then 4 C overnight. The globulins were precipitated with 40% saturated ammonium sulfate $(NH_4)_2SO_4$ at 4 C and centrifuged in the cold. One half of the 200 µl centrifuged mixture (supernatant) was removed and both this fraction and the remaining precipitate containing fraction were counted in a Beckman Biogamma counter (Fullerton, Ca.). The percent radiolabeled SACHO antibody is equal to:

$$\frac{\text{cpm in precipitate} - \text{cpm in supernatant}}{\text{cpm in precipitate} + \text{cpm in supernatant}} \times 100$$

To determine the level of anti-SACHO antibody the percent SACHO bound by the unknown sample was compared with the percent SACHO bound by serial dilutions of a standard amount of anti-SACHO antibody. Each unknown serum sample was assayed at least in duplicate.

Levels of precipitating anti-SACHO antibodies were determined by reverse radial immunodiffusion (RRID) as has been previously described (Leslie and Hattier 1974).

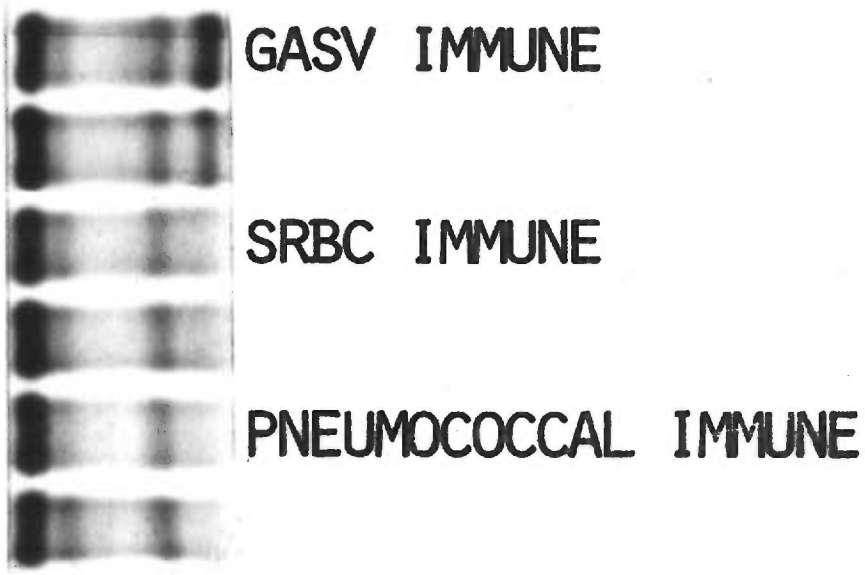
CHARACTERIZATION OF ID-1

Analysis of immune responsiveness of rats to GASV

Hyperimmunization of rats by iv injection of a pepsin digest of heat killed Group A streptococcal strain J17A4 (1 mg/ml, 3 times per week for 3 weeks) induced high levels of anti-SACHO antibodies of restricted heterogeneity. This is represented in Figure 1 by cellulose acetate electrophoretic patterns of sera from GASV immune rats as compared with rats immunized via the same protocol with a 10% concentration of sheep red blood cells (SRBC) or $5x10^9$/ml Type S_8 pneumococcal bacteria. The restriction in the immune response of rats to GASV has allowed for serological identification of an idiotype associated with anti-SACHO antibodies (Id-1). In individual sera the percentage of anti-SACHO antibodies possessing Id-1 determinants varies from 0 to >90%. Anti-SACHO antibodies used for the preparation of anti-idiotypic antibodies and as reference standards in the Id-1 assay possess Id-1 on greater than 90% of their anti-SACHO antibody molecules.

To prepare an anti-idiotypic reagent, 2 rabbits were immunized with the $F(ab')_2$ fragment of specifically purified anti-SACHO antibodies of an individual rat (5-1 IgG). $F(ab')_2$ fragments were prepared by digesting 5-1 IgG with 1% (w/w) pepsin for 12 hours in 0.1 M acetate buffer pH 4.5. Digestion was stopped by de-acidifying with 0.1 M NaOH. $F(ab')_2$ fragments were separated by filtration over a Sephadex G-150 column calibrated with normal rabbit IgG, $F(ab')_2$ and Fab' fragments. Rabbits were initially immunized subcutaneously at

Figure 1. Cellulose acetate electrophoretic patterns of sera from HPR rats immunized iv with 1 mg/ml GASV, 10% concentration of SRBC, or $5x10^9$/ml Type 8 pneumococcal bacteria. The concentration of SRBC and pneumococci injected were comparable in dry weight to the amount of GASV injected.

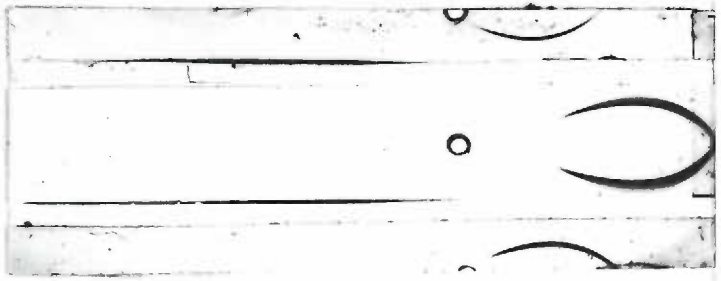

GASV IMMUNE
SRBC IMMUNE
PNEUMOCOCCAL IMMUNE

multiple sites with 500 µg of 5-1 $F(ab')_2$ mixed with an equal volume of Freund's complete adjuvant (FCA). Two weeks later rabbits were given 500 µg of the same immunogen mixture. A bleeding from both animals taken 4 weeks after the initial exposure to antigen gave positive reactions specifically with Id-1 preparations as determined by Ouchterlony analysis and immunoelectrophoresis (IE). One rabbit died after 2 months of immunization; however, the second one was subsequently bled at weekly or biweekly intervals for 6 months. During this time the rabbit was re-immunized periodically with 5-1 $F(ab')_2$ in Freund's incomplete adjuvant (FIA) to maintain high antibody titers. Antisera were analyzed by IE and pooled based upon their IE reactivity and collection dates. The individual antiserum pools were made Id-1 specific by passage over normal rat serum, normal rat IgG, and normal rat $F(ab')_2$ or Fab immunoabsorbent columns. The IgG fraction was obtained by precipitation with 40% saturated ammonium sulfate prior to Sephadex G-200 gel filtration. IE analysis of 6 different anti-Id-1 preparations are shown in Figures 2 and 3. The idiotype, as defined by anti-5-1 $F(ab')_2$ antiserum, appeared to be identical to the cross-reactive idiotype associated with anti-SACHO antibodies previously identified by Stankus and Leslie (1974).

Quantitation of Id-1

Id-1 was quantified by a competitive inhibition radioimmunoassay (RIA) using the method of Kindt et al. (1972). Specifically, 680 ng of purified anti-Id-1 in a 50 µl volume was added to unknown serum samples diluted in a 50 µl volume. The buffer used for all dilutions

Figure 2. Immunoelectrophoretic analysis of early preparations of anti-Id-1 antisera from rabbits 1 and 2.



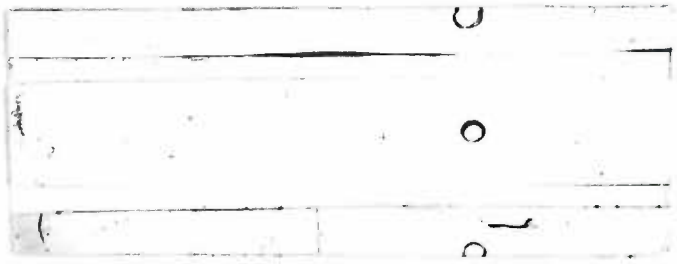
ID-1+ 5-1

ANTI-ID-1---RABBIT 1

ID-1+ 5-1 $F(AB')_2$

ANTI-ID-1---RABBIT 2

ID-1+ 5-1

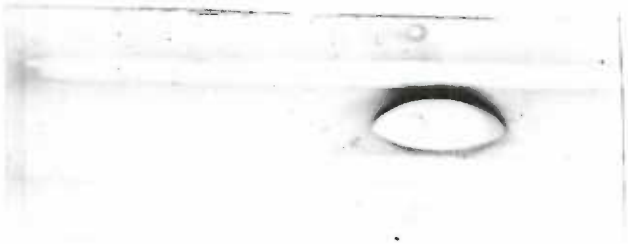


ANTI-ID-1---RABBIT 1

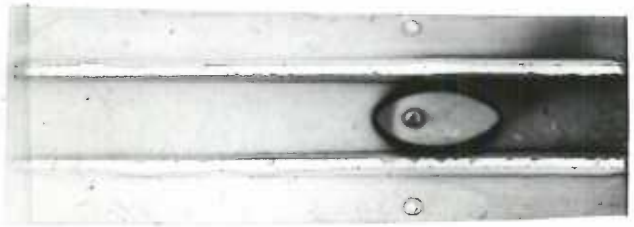
NORMAL HPR RAT SERUM

ANTI-ID-1---RABBIT 2

Figure 3. Immunoelectrophoretic analysis of 4 later preparations of anti-Id-1 antisera.



NORMAL HPR RAT SERUM
ANTI-ID-1---PREPARATION A
ID-1+ 6-5
ANTI-ID-1---POOL 1
NORMAL HPR RAT SERUM

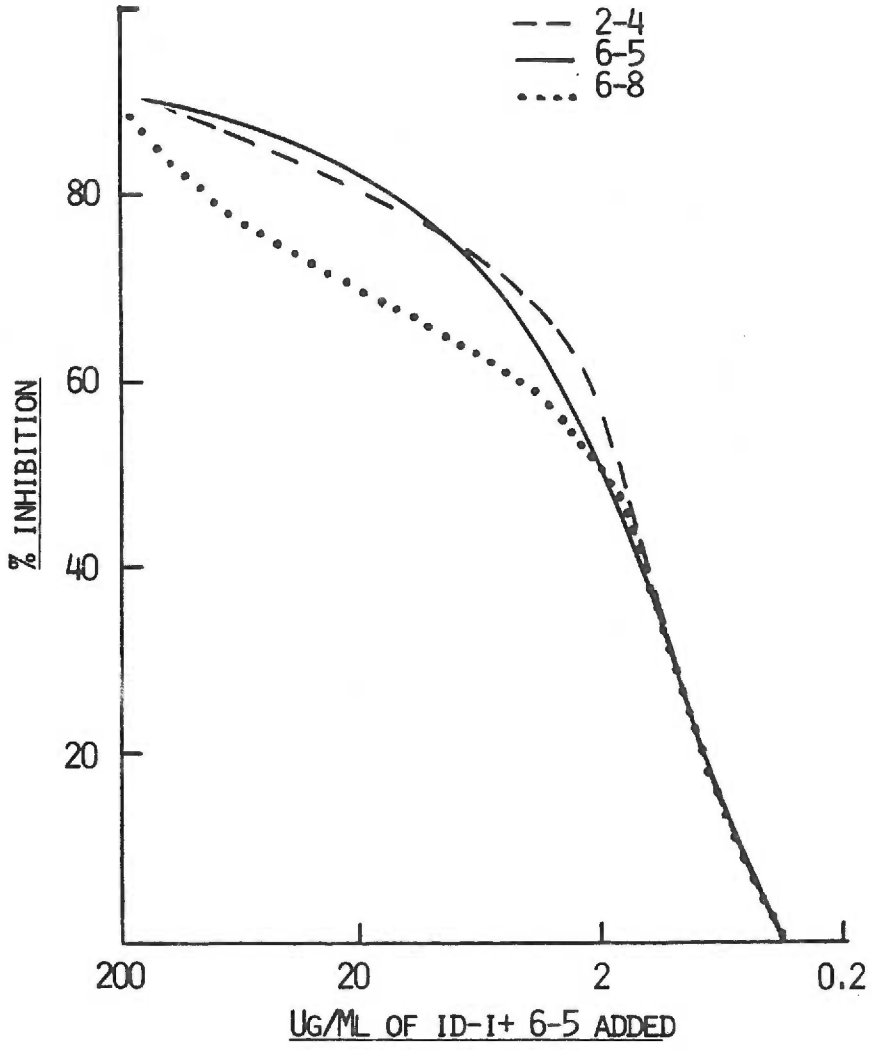


HPR IGG 2C+ RAT IG
ANTI-ID-1---RABBIT 1
ID-1+ 6-5
ANTI-ID-1---RABBIT 2
HPR IGG 2C+ RAT IG

was a 0.01 M phosphate buffered saline, pH 7.0, to which 1% ovalbumin and a 1:50 dilution of normal rat serum were added. Approximately 290 ng in 50 µl of ^{125}I radiolabeled Id-1 positive anti-SACHO preparation (6-5 IgG) was added to the anti-Id-1 serum sample mixture, which was then incubated at 37 C for 1 hour. To precipitate the immune complex, 50 µl of a 1:4 dilution of sheep anti-rabbit IgG antiserum (PCL-RIA; Lot #21-30-40-1) was added to each tube. The use of radiolabeled Id-1 positive antibody molecules from a different individual rat (6-5 IgG) allowed for more efficient detection and quantitation of the cross-reactive idiotype. Levels of Id-1 were quantified relative to a standard dilution curve generated by dilutions of 6-5 IgG, which has greater than 90% Id-1 positive molecules. The sensitivity of the RIA relative to 6-5 IgG was 20 ng per sample.

When 3 different radiolabeled preparations of Id-1^+ IgG were analyzed relative to dilutions of 6-5 IgG standard, one of the radiolabeled preparations (6-8) produced a standard curve with a different slope when compared with the other 2 Id-1^+ preparations (Figure 4). All three preparations, however, can bind the same anti-Id-1 antiserum preparation with greater than 90% efficiency. This suggests that Id-1, as defined by the anti-Id-1 antiserum, represents more than a single determinant and that the anti-Id-1 antiserum serves as a tool allowing for the recognition of a family of closely related anti-SACHO antibody molecules. The dissimilarity between Id-1^+ 6-8 and either 2-4 or 6-5 is also represented by cellulose acetate electrophoretic profiles (Figure 8). Absorption of anti-Id-1 by different Id-1^+ preparations,

Figure 4. Three different ^{125}I radiolabeled Id-1^+ preparations were analyzed for their effectiveness in inhibiting the binding of Id-1^+ 6-5 to anti-Id-1.


2-4
6-5
6-8
80
60
40
20
% INHIBITION
200
20
2
0.2
UG/ML OF ID-I+ 6-5 ADDED

however, resulted in the removal of all precipitating anti-Id-1 reactivity with Id-1^+ sera samples, suggesting that one cannot specifically absorb the precipitating reactivity of a subpopulation of anti-Id-1 antibody molecules.

Characterization of anti-Id-1 reactivity

Unlike results previously reported by Stankus and Leslie (1974), the incorporation of either 0.15 M or 0.05 M hapten (N-acetylglucosamine; N-AcGln) into agar did not (by Ouchterlony analysis) inhibit precipitation of Id-1^+ preparations with anti-Id-1 antiserum prepared against 5-1 $F(ab')_2$ IgG fragments. By RIA analysis, 0.3 M N-AcGln inhibited Id-1 interaction with anti-Id-1 by 46.7%. This compares with no inhibition of reactivity by 0.3 M glucosamine-HCl and <10% inhibition by 0.3 M glucose or 0.3 M galactose. N-Acgln at a 0.15 M concentration produced 38.7% inhibition. The addition of SACHO and GASV, at concentrations as high as 1 mg/ml, were capable of inhibiting the binding of Id-1 to anti-Id-1 by 30-40%. Thus a portion of Id-1-anti-Id-1 interactions are hapten inhibitable, suggesting that anti-Id-1 antiserum has specificity for determinants either directly associated with, or in close proximity to the antibody combining site. The lack of complete hapten inhibition in RIA analysis, and the lack of hapten inhibition of precipitation reactivity between Id-1 and anti-Id-1 indicate that the anti-Id-1 antiserum recognized additional determinants unique to anti-SACHO antibody molecules, but not intimately involved in antigen recognition. This is consistent with N-terminal amino acid sequencing analysis of anti-SACHO heavy chain (H-chains).

Anti-SACHO H-chains not only appeared to represent a minor subgroup, the V_HIII subgroup, Id-1$^+$-anti-SACHO molecules also have a very unusual lysine substitution at position 3 (Klapper et al. 1976). These observations lead to the interesting speculation that there is a linkage between expression of this rare framework region and the hypervariable region associated with Id-1 expression.

To determine if Id-1 complexed with anti-Id-1 could be detected in the Id-1 RIA, Id-1$^+$ anti-SACHO molecules at concentrations ranging from 0.5-80 μg/ml were mixed with a constant amount of heterologous anti-Id-1 [antigen binding capacity (ABC)=40 ng], and the percent inhibition produced by this mixture was compared to that produced by the same concentration of Id-1 alone. At higher concentrations of Id-1 (60-80 μg/ml) the Id-1-anti-Id-1 mixture and Id-1 alone were equally effective in inhibiting the Id-1 RIA. At lower Id-1 concentrations (0.5-1 μg/ml), the Id-1-anti-Id-1 mixture was an 8-16% less effective inhibitor than the same concentration of Id-1 alone. This implies that by RIA analysis the concentration of Id-1 detectable in animals which produce Id-1 and anti-Id-1 simultaneously is lower than that actually present. The error present in such quantifications is more apparent at low Id-1 to anti-Id-1 ratios.

Recent studies have identified a carbohydrate moiety associated with the second hypervariable region of anti-α-1-3-dextran antibodies (Clevinger et al. 1980). In order to eliminate the possibility that anti-Id-1 has anti-carbohydrate reactivity,(specifically anti-N-AcGln), it was found that passage of anti-Id-1 over an N-AcGln immunoadsorbent did not remove its Id-1 reactivity. In addition, if anti-Id-1 was

incubated with concentrations of 0.5-10 mg/ml of GASV, centrifuged, and the supernatant used as the source of anti-Id-1 in the Id-1 RIA, prior incubation with GASV did not affect the Id-1 binding capacity of the anti-Id-1 antiserum.

Since the assay for Id-1 is a competitive inhibition RIA, in theory it is possible that autologous anti-Id-1 could inhibit ^{125}I radiolabeled Id-1 binding to rabbit anti-Id-1, just as autologous Id-1 inhibits such binding. To determine if the Id-1 RIA distinguished between Id-1 and anti-Id-1, sera suspected of containing auto-anti-Id-1 were passaged over either an Id-1^+, anti-Id-1^+, and/or over N-Acgln immunoadsorbent columns and analyzed for Id-1 levels by RIA. Sera samples analyzed included those from rats extensively immunized with Id-1 to induce autologous anti-Id-1, and those from animals neonatally injected with heterologous anti-Id-1 to induce subsequent suppression of Id-1 synthesis. Such analysis verified that there was no preferential binding of the component detectable by Id-1 RIA to the Id-1 immunoadsorbent, implying that enrichment for anti-Id-1 did not enrich for Id-1 RIA analyzable reactivity. Also, it has been observed that in all sera analyzed, Id-1 reactivity is selectively removed by passage over an N-AcGln immunoadsorbent column, and eluted from the column by NaSCN. Thus, autologous anti-Id-1, if produced, cannot effectively compete with heterologous anti-Id-1 in this RIA.

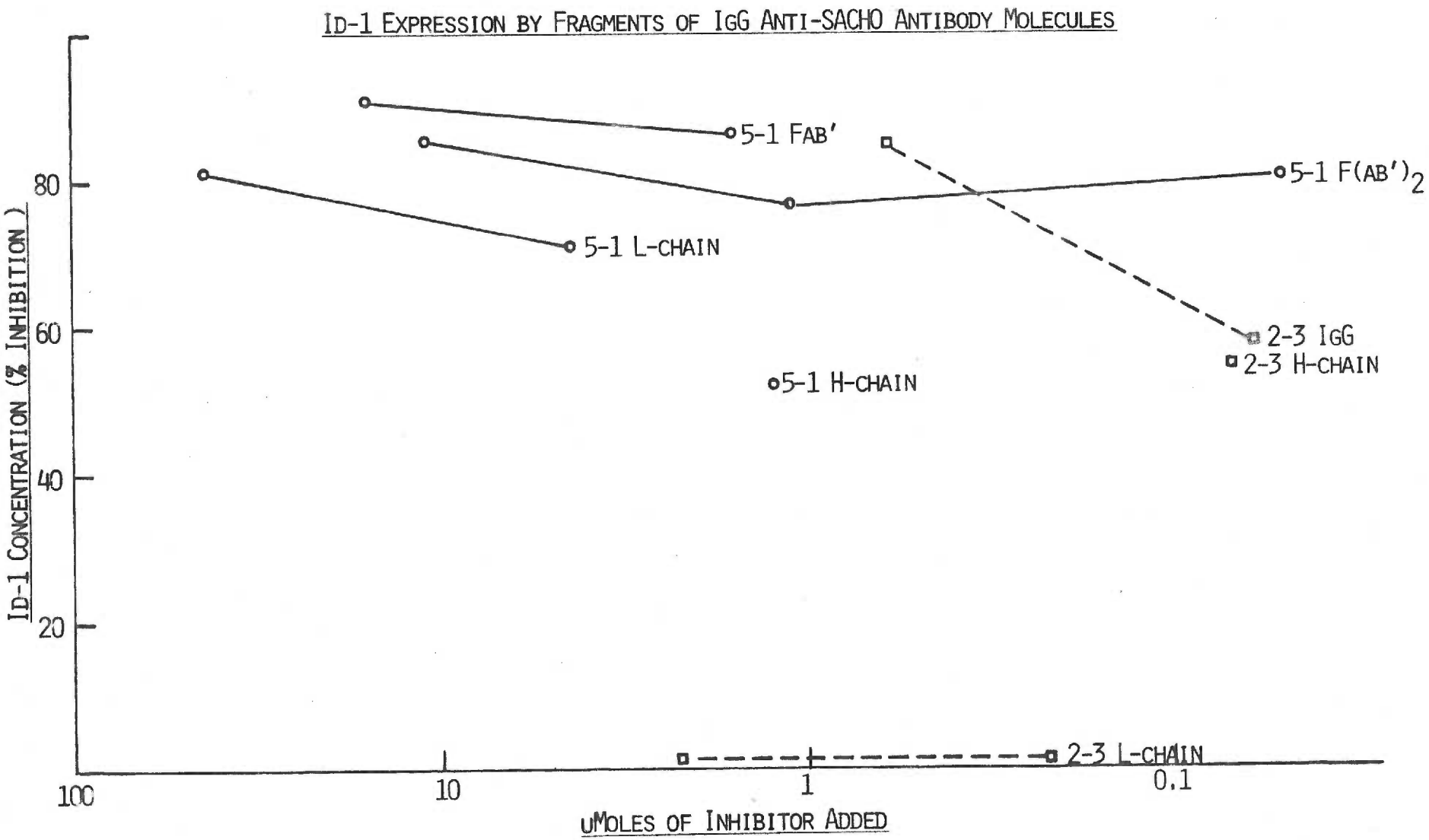
Localization of Id-1 determinants

In order to determine which portion of the anti-SACHO antibody molecule expressed Id-1 determinants, specifically purified IgG

anti-SACHO antibodies were fractionated into $F(ab')_2$ and Fab fragments, and into individual heavy and light (H and L) chains. $F(ab')_2$ and Fab fragments of anti-SACHO IgG and normal rat IgG were prepared as described earlier and separated by Sephadex G-150 gel filtration. H and L chains were prepared by partial reduction with 0.1 M dithioerythritol at room temperature for 1 hour, followed by alkylation with 0.22 M iodoacetamide at 4 C for 1 hour. The presence of Id-1 determinants on each of the antibody fragments was determined by their ability to effectively inhibit binding of ^{125}I Id-1 IgG to anti-Id-1.

As shown in Figure 5, Id-1^+ IgG molecules were the most efficient inhibitors in the Id-1 RIA. Although the $F(ab')_2$ or Fab fragments did not inhibit as efficiently on a molar basis as did the IgG molecule, they were both effective inhibitors, indicating that Id-1 determinants are associated predominantely with the combining site portion of the anti-SACHO molecule rather than the Fc portion. It is evident from RIA analysis of Id-1^+ H- and L-chains that both chains are required for complete expression of Id-1. The lack of complete inhibition by H- and L-chains, as well as that by the antibody fragments stresses the importance of teritary and quaternary structures for serological recognition of Id-1 determinants by this anti-Id-1 antiserum. The possibility that either the H or the L-chain maintains the more immunodominant determinants requires further analysis since the contribution of the individual chains varies between the 2 different anti-SACHO antibody preparations analyzed.

Figure 5. Id-1 expression by fragments of IgG anti-SACHO antibody molecules.


ID-1 EXPRESSION BY FRAGMENTS OF IgG ANTI-SACHO ANTIBODY MOLECULES
ID-1 CONCENTRATION (% INHIBITION)
80
60
40
20
5-1 FAB'
5-1 F(AB')2
5-1 L-CHAIN
2-3 IgG
2-3 H-CHAIN
5-1 H-CHAIN
2-3 L-CHAIN
100
10
1
0.1
uMOLES OF INHIBITOR ADDED

Inheritance of Id-1 Expression

It was the purpose of these studies to determine if a linkage exists between Id-1 expression or the degree of Id-1 expression and the genetic background of individual animals. Previously, Stankus and Leslie detected Id-1 production in nine different strains of inbred rats following immunization with GASV. These nine strains were representative of 8 defined Ag-B (major histocompatibility antigens of the rat) haplotypes and the 4 defined immunoglobulin allotypes in rats, strongly suggesting the lack of linkage between major histocompatibility haplotypes, allotypes and Id-1 expression. Although all immunized strains produced Id-1, the percentage of anti-SACHO antibodies possessing Id-1 varies from 0 through >90% depending upon the individual animal. In addition, previous studies in our laboratory by Stankus showed that although the ability of rats to produce either high or low levels of precipitating anti-SACHO was an inheritable trait, there appeared to be no genetically simple pattern of inheritance of Id-1 expression. As represented in Figure 6, when monitoring Id-1 expression of rats selectively inbred for high anti-SACHO responsiveness, the percentage of Id-1^+ offspring remained at 75% (F_3 generation) after 2 generations of mating Id-1^- animals. Figure 6 monitors Id-1 expression in animals selectively inbred for low responsiveness. Again, no apparent relationship exists between the presence or absence of Id-1 production by parents and the apparent percentage of Id-1^+ progeny.

Figure 6. Id-1 expression by rats selective inbred for high and low levels of precipitating antibodies (■ , ● Id-1^+ ♂'s and ♀'s, respectively; □ , ○ - Id-1^- ♂'s and ♀'s, respectively).

High anti-SACHO producers

P: Parental ppt. anti-SACHO = 2.65 mg/ml.

F_1: Parental ppt. anti-SACHO = 1.75 mg/ml.

F_2: Parental ppt. anti-SACHO = 6.65 mg/ml.

F_3: Parental ppt. anti-SACHO = 19.9 mg/ml.

Low anti-SACHO producers

P: Parental ppt. anti-SACHO $\bar{x}$ = 0.4 mg/ml.

F_1: Parental ppt. anti-SACHO $\bar{x}$ = 0.50 mg/ml.

F_2: Parental ppt. anti-SACHO $\bar{x}$ = 0.25 mg/ml.

F_3: Parental ppt. anti-SACHO x = 0.70 mg/ml.

F_4: Parental ppt. anti-SACHO $\bar{x}$ = 0 mg/ml.

Inbred for High Levels of Anti-SACHO Antibodies

% Id-I+

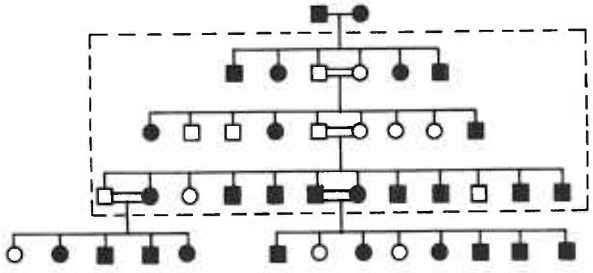
P : 100

F_1 : 67

F_2 : 33

F_3 : 75

F_4 : 77



Inbred for Low Levels of Anti-SACHO Antibodies

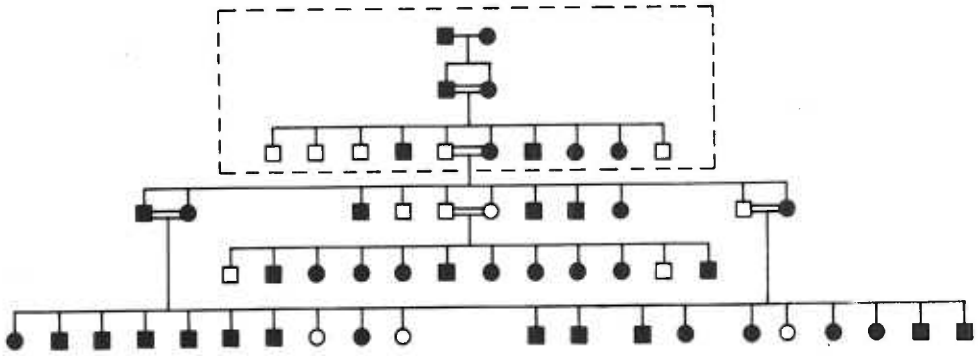
P : 100

F_1 : –

F_2 : 50

F_3 : 63

F_4 : 84



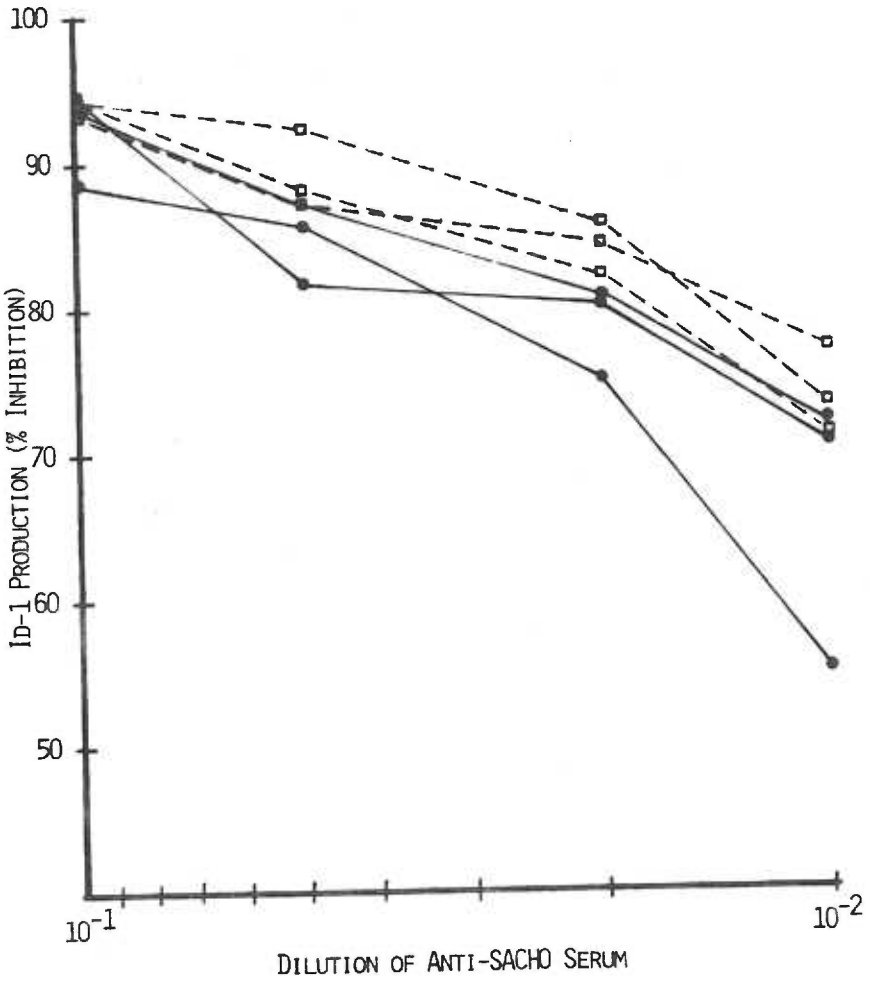
Recently, Leslie (1979) observed that rat anti-SACHO antibody molecules were predominantely of the IgG_{2c} subclass, which normally represents a minor subclass of immunoglobulins produced by the rat. Cross immunizations between different strains of rats resulted in the production of an antiserum identifying an IgG_{2c} allotype. The prototype inbred strain possessing this allotype is the Copenhagen (AgB:4, Kappa:1b, IgG_{2b}:1b) and the anti-allotype antiserum was made in SHR rats (AgB:8, Kappa:1b, IgG_{2b}:2b). Id-1 determinants were not part of the IgG_{2c} allo-determinant as was evidenced by the presence of precipitable IgG_{2c} allotype in Copenhagen pre-immune sera, but the lack of detectable Id-1 by RIA in both pre-immune SHR and Copenhagen sera.

In order to determine if a linkage exists between this serologically defined IgG_{2c} allele and Id-1 expression, (SHRxCopenhagen)F_1 rats were backcrossed with SHR male rats. If Id-1 expression was linked to expression of the Copenhagen allotype, those rats lacking the allotype should have altered expression Id-1 when compared with those possessing the IgG_{2c} allotype. Id-1 levels and allotype production by (CopenhagenxSHR)xSHR backcrossed rats 2 weeks after GASV immunization is shown in Table I. No direct correlation between levels of Id-1 production and expression of the IgG_{2c} allotype was observed. In addition, when increasing dilutions of IgG_{2c} allotype positive and allotype negative anti-SACHO sera were assayed in the Id-1 RIA, the slopes of the dilution curves were very similar, regardless of the expression or lack of expression of the allotype (Figure 7).

Table 1. Id-1 levels and IgG_{2c} allotype expression by (SHR x Copenhagen) x SHR backcross rats

Animal Number	Id-1 level (μg/ml)	IgG_{2c} SHR anti-Copenhagen Allotype
♂3-7	7	-
♀4-7	11	+
♀3-5	47	+
♂3-11	50	+
♂3-10	90	+
♂3-3	108	+
♂4-2	125	+
♂3-12	165	+
♂3-9	225	-
♀3-4	230	-
♂4-1	235	+
♀3-6	265	-
♂3-2	273	+
♂3-1	298	+
♂4-5	340	-
♀4-10	350	+
♀4-4	390	+
♀4-6	535	+

Figure 7. Id-1 analysis of IgG_{2c} allotype positive (_____) and allotype negative (-----) anti-SACHO sera.


100
90
80
70
60
50
ID-1 PRODUCTION (% INHIBITION)
10^{-1}
10^{-2}
DILUTION OF ANTI-SACHO SERUM

Thus, although allotypic differences in these molecules were apparent, at least by this criteria of analysis, these differences were not reflected at the idiotypic level.

Previous studies in mice have shown that there is a linkage between H-chain allotype and idiotype expression (Eichmann 1973, Krause 1974, Weigart and Potter 1977, Cancro et al. 1978). Using the above criteria of analysis, a linkage between Id-1 expression and the IgG_{2c} allotype of rat anti-SACHO was not readily apparent. One, however, cannot rule out the possibility that such a linkage might become evident through analysis with monoclonal antibodies.

Analysis of the class and subclass distribution of $Id\text{-}1^+$ and $Id\text{-}1^-$ anti-SACHO antibodies

As mentioned earlier, both anti-SACHO and Id-1 are associated predominately with the IgG_{2c} subclass of rat immunoglobulin molecules. However, as is apparent from Table II, sera obtained from GASV immunized HPR and F344 rats had, in addition to higher IgG_{2c} levels, consistently higher levels of IgM and, in general, higher levels of IgG_1, IgG_{2a} and IgG_{2b} than did sera from non-immune animals. This suggests that either a significant portion of anti-SACHO antibodies are associated with IgM and the other IgG subclasses of immunoglobulins, or that immunization with GASV stimulated immunoglobulin production by other than N-Acetylglucosamine specific B cell clones. Also, it is observed from Table II that there are differences in levels of production of the various immunoglobulin classes or subclasses depending upon the presence or absence of Id-1 production by the individual

Table 2. Id-1, anti-SACHO and immunoglobulin class and subclass levels of Id-1$^+$ and Id-1$^-$ sera

		Id-1	Anti-SACHO		IgM	IgG1	IgG2a	IgG2b	IgG2c	IgA
HPR		mg/ml	RIA	RID			(mg/ml)			
Normal serum		0		0	1.8	1.4	3.3	6.0	0.24	0.33
Id-1$^+$	2-4	>40.0		35.0	16.5	2.5	2.9	7.3	≈21.0	0.25
	6-5	>40.0		83.0	8.5	1.8	3.9	7.2	≈26.0	0.48
	6-8	22.0		39.0	3.2	1.62	5.8	7.7	≈25.0	0.37
$\bar{x}$±SEM				52.3	9.4	1.97	4.2	7.4	24.0	0.37
				±15.4	±3.9	±0.27	±0.9	±0.15	±1.5	±0.07
Id-1$^-$	1-4	0.01		6.3	4.4	1.8	6.0	15.2	5.2	1.2
	1-1	<0.01		1.2	7.0	2.6	6.0	14.4	4.0	1.5
	7-2	0.02		0	10.0	2.75	6.8	28.8	2.8	0.5
$\bar{x}$ SEM				2.5	7.1	2.38	6.3	19.5	4.0	1.07
				±1.9	±1.6	±0.29	±0.27	±4.7	±0.7	±0.30
F344										
Normal serum		0	0	0	0.87	0.35	0.69	3.06	0.15	0.13
Id-1$^+$	4-1	0.95		0	3.2	0.33	8.0	>6.5	1.2	0.06
	4-2	0.92		0	3.2	0.25	3.7	4.5	0.8	0.05
	4-3	1.69		0	6.5	0.47	5.8	5.0	0.7	0.12
	4-4	1.17		0	3.7	0.28	5.8	6.5	0.6	0.08
	4-5	0.62		0	3.2	0.25	2.9	>7.0	0.76	0.12
Pooled sera		0.12		0	2.4	1.4	>10.0	4.0	0.8	0.48
$\bar{x}$±SEM				0	3.7	0.50	6.0	5.6	0.81	0.15
					±0.6	±0.18	±1.1	±0.5	±0.08	±0.07
Id-1$^-$	5-3	0		0	3.2	0.78	7.8	7.0	0.5	0.37
	5-4	0.009		0	3.5	0.74	>10.0	>8.0	0.53	0.33
$\bar{x}$±SEM				0	3.35	0.76	8.9	7.5	0.52	0.35
					±0.15	±0.02	±1.1	±0.5	±0.02	±0.02

animal. For example, Id-1^+ animals of both the HPR and F344 strains consistently produced higher levels of IgG_{2c}, whereas Id-1^- animals produced higher levels of IgG_{2b} and IgA, and had a tendency for higher production of IgG_1 and IgG_{2a}.

In order to analyze the proportion of the anti-SACHO antibodies represented by each of the classes or subclasses, Id-1^+ and Id-1^- sera from individual animals were specifically purified by passage over the hapten, N-acetylglucosamine, immunoadsorbent column, and eluted in an identical manner, sequentially, by temperature shift, hapten, or increasing molarities of NaSCN. The different elution methods were utilized in order to gain a further understanding of class or subclass distribution of anti-SACHO antibodies as related to their avidity for the hapten conjugated column. In Table III are represented the mg of different immunoglobulin classes or subclasses per mg of protein which were eluted from the hapten column by the indicated method. It is apparent that specifically purified anti-SACHO antibodies obtained from the combined methods of elution, as defined by this protocol, are predominately of the IgM class or IgG_{2c} subclass. It is also apparent that the increase in levels of the other Ig classes in immune sera cannot always be accounted for by a proportionate increase in antibodies having specificity for N-AcGln. It is possible that these immunoglobulins may have specificity for the rhamnose backbone or other antigenic determinants on the surface of Group A streptococci, or that they actually are a result of a non-specific stimulation. It is also of interest to determine if these apparent non-hapten specific immunoglobulins possess Id-1 determinants.

Table 3. Immunoglobulin class and subclass distribution of specifically purified anti-SACHO antibodies from Id-1$^+$ and Id-1$^-$ HPR rats

	Protein conc. (mg/ml)	Id-1	Anti-SACHO RIA	Anti-SACHO RID	IgM	IgG1	IgG2a	IgG2b	IgG2c	IgA
Id-1$^+$ HPR										
2-4 serum[a]	-	>40.0		35.0	16.5	2.5	2.9	7.3	≈21.0	0.25
RT Tris	1.1	>3.6[b]		0.26	0.63	0.15	0	0.33	0.26	0
Hapten	10.0	2.3		1.2	0.09	<0.02	<0.01	<0.02	0.58	0
1M NaSCN	0.2	1.07		0	0.5	0	0	0	0.2	0
3M NaSCN	0.16	0.03		0	0	0	0	0	0.18	0
6-5 serum	-	>40.0		>40.0	8.5	1.8	3.9	7.2	≈26.0	0.48
RT Tris	45.0	>0.89		1.33	0.04	<0.01	<0.01	<0.01	0.44	<0.01
Hapten	16.0	0.17		1.59	0.07	0	<0.01	0.02	0.4	0
1M NaSCN	20.0	>2.0		0.20	0.08	<0.01	<0.01	<0.01	0.33	<0.01
3M NaSCN	2.0	0.22		0.90	0.95	0	0	0	0.35	0.04
6-8 serum	-	22.0		39.0	3.2	1.62	5.8	7.7	≈25.0	0.37
RT Tris	12.9	>3.1		0.7	0.04	<0.01	<0.01	0.02	1.4	<0.01
Hapten	17.4	>2.3		0.15	0.21	<0.01	<0.01	<0.01	0.14	0
1M NaSCN	2.0	0.05		0.15	0	0	0	0	0.2	0
3M NaSCN	0.9	0.11		0.33	0	0	0	0	<0.04	0
$\bar{x}$Hapten Elution Proportion:		>1.59 ±0.71		0.98 ±0.43	0.12 ±0.04	<0.01 ±0.006	<0.01 ±0	<0.02 ±0.003	0.37 ±0.12	0
$\bar{x}$Combined elution Proportions:		>1.32 ±0.37		0.57 ±0.16	0.22 ±0.09	<0.02 ±0.01	<0.005 ±0.001	<0.04 ±0.03	0.38 ±0.10	<0.005 ±0.003

Table 3. (Continued)

	Protein conc. (mg/ml)	Id-1	Anti-SACHO RIA	Anti-SACHO RID	IgM	IgG1	IgG2a	IgG2b	IgG2c	IgA
Id-1⁻ HPR										
1-4 serum	-	0.01		6.3	4.4	1.8	6.0	15.2	5.2	1.2
RT Tris	5.3	0		0	1.06	0.02	0.06	0.26	0.14	0.01
Hapten	6.5	<0.01		0.81	0.43	<0.02	<0.02	0.02	0.97	0
1M NaSCN	1.7	<0.01		0.41	0.23	0	<0.06	<0.06	0.55	0.06
3M NaSCN	2.1	<0.01		<0.09	0.43	0	<0.05	<0.05	0.19	0.24
1-1 serum	-	<0.01		1.2	7.0	2.6	6.0	14.4	4.0	1.53
RT Tris	3.6	0		0	0.72	0.05	0.06	0.36	0.14	0.17
Hapten	5.3	<0.01		0.32	0.04	<0.01	<0.02	0.02	0.73	0.04
1M NaSCN	1.2	<0.01		1.8	0.25	0	<0.08	<0.08	0.63	<0.02
3M NaSCN	1.5	<0.01		0	0.13	0	<0.07	<0.07	0.03	0
7-2 serum	-	0.02		0	10.0	2.75	6.8	28.8	2.8	0.48
RT Tris	1.8	0		0	0.56	0.05	<0.06	0.17	0.36	<0.06
Hapten	9.4	<0.01		0.85	0.13	<0.01	<0.01	<0.01	0.60	<0.01
1M NaSCN	4.7	0.01		0.21	0.06	<0.01	<0.02	<0.02	0.34	0
3M NaSCN	3.9	<0.01		0.31	0.05	<0.01	<0.03	<0.03	0.36	0
$\bar{x}$Hapten Elution Proportions		<0.01 ±0		0.66 ±0.17	0.20 ±0.12	<0.01 ±0.003	<0.02 ±0.003	0.02 ±0.003	0.77 ±0.11	0.02 ±0.01
$\bar{x}$Combined Elution Proportions		<0.008 ±0		0.4 ±0.07	0.34 ±0.10	<0.02 ±0.003	<0.04 ±0.009	<0.07 ±0.02	0.42 ±0.02	<0.05 ±0.02

[a]Serum antibody and Ig levels: mg/ml serum.

[b]Fractions eluted from N-AcGlN IA: mg/mg protein.

Tables III and IV indicate that essentially the same proportion of IgG_{2c} anti-SACHO molecules are produced by either Id-1^+ or Id-1^- GASV immunized rats. Although there was a slight tendency for Id-1^- anti-SACHO antibodies to have higher proportions of the IgA class, in general there were no pronounced differences in the class or subclass distribution of anti-SACHO antibody molecules and the expression or lack of expression of Id-1 by HPR rats. Therefore by this method of analysis, there appears to be no linkage between the expression of Id-1 and immunoglobulin class or subclass distribution of anti-SACHO antibodies.

In order to determine if Id-1 could be linked to immunoglobulins of other than the IgG_{2c} subclass, Id-1 preparations were extensively examined by Ouchterlony analysis. Such studies showed that some Id-1^+ preparations produced a line of partial identity when reacted with anti-Id-1 and anti-IgG_{2b} in juxtaposition. In addition, at the cell surface level, it was shown by fluorescent staining of B lymphocytes that both IgM and IgD are associated with Id-1 (unpublished observation).

Further analysis of Id-1^+ and Id-1^- sera and specifically purified anti-SACHO by cellulose acetate electrophoresis (Fig. 8) indicated that both Id-1^+ and Id-1^- antibodies exhibited restricted heterogeneity, implying production of restricted anti-SACHO antibodies is not necessarily synonymous with production of Id-1. The production of Id-1^- anti-SACHO antibodies of restricted heterogeneity provides one with excellent tools for preparing anti-Id-2, or anti-Id-3, etc.,

Table 4. Immunoglobulin class and subclass distribution of specifically purified anti-SACHO antibodies from Id-1$^+$ and Id-1$^-$ F344 rats

	Protein conc. (mg/ml)	Id-1	Anti-SACHO RIA	Anti-SACHO RID	IgM	IgG1	IgG2a	IgG2b	IgG2c	IgA
Normal F344 serum[a]		0		0	0.87	0.13	0.6	3.06	0.06	0.1
Id-1$^+$ sera										
4-1		0.95		0	3.2	0.33	8.0	>6.5	1.2	0.06
4-2		0.92		0	3.2	0.25	3.7	4.5	0.8	0.05
4-3		1.69		0	6.5	0.47	5.8	5.0	0.7	0.12
4-4		1.17		0	3.7	0.28	5.8	6.5	0.6	0.08
4-5		0.62		0	3.2	0.25	2.9	>7.0	0.76	0.12
$\bar{x}$±SEM		1.07		0	4.0	0.32	5.2	5.9	0.81	0.09
		±0.18			±0.64	±0.04	±0.9	±0.48	±0.10	±0.01
Id-1$^+$ Serum pool #1		0.12		0	2.4	1.4	>10.0	4.8	0.8	0.48
RT-TR15	2.3	0.01[b]		0	0.64	0.013	0.13	0.18	0.13	0.03
1M NaSCN	3.1	0.09		0	1.03	0.008	0.06	0.11	0.26	0.03
2M NaSCN	2.2	0.23		0.23	1.45	0	<0.05	0.05	0.09	<0.01
$\bar{x}$		0.11		0.08	1.04	0.007	0.08	0.11	0.16	0.02
Id-1$^+$ Serum pool #2										
RT-TR15	1.9	0.07		0	0.66	0.01	0.09	0.22	0.11	0
1M NaSCN	1.2	0.68		0	0.63	0	<0.04	<0.08	0.3	0
3M NaSCN	2.3	0.03		0	0.22	0	<0.02	<0.04	0.43	0
$\bar{x}$		0.26		0	0.50	0.003	<0.05	0.11	0.28	0
$\bar{x}$±SEM		0.19		0.04	0.77	0.005	0.07	0.12	0.22	0.01
		±0.11		±0.04	±0.19	±0.003	±0.02	±0.03	±0.06	±0.007

Table 4. (Continued)

	Protein conc. (mg/ml)	Id-1	Anti-SACHO RIA	Anti-SACHO RID	IgM	IgG1	IgG2a	IgG2b	IgG2c	IgA
Id-1⁻ 5-3 serum		0		0	3.2	0.78	7.8	7.0	0.5	0.37
RT TR15	0.89	0		0	0.08	0.03	0.11	0.56	0.04	0
Hapten	0.54	0		0	0.07	0	<0.19	<0.19	<0.04	0
1M NaSCN	0.57	0		0	0.12	0	<0.18	<0.18	<0.04	0.14
3M NaSCN	0.4	0		0	0.18	0	<0.25	<0.25	<0.05	0.45
$\bar{x}$		0		0	0.12	<0.01	<0.18	<0.30	<0.04	0.15
Id-1⁻ 5-4 serum		0.009		0	3.5	0.74	>10.0	>8.0	0.53	0.33
RT-TR15	1.1	0		0	0.13	0.02	0.09	0.18	0.03	0.09
Hapten	0.5	0.01		0	0.78	0	<0.2	<0.2	0	0
1M NaSCN	0.6	<0.01		0	0.15	0	<0.17	<0.17	0	0.13
3M NaSCN	0.7	0		0	0	0	<0.14	0	0	0.11
$\bar{x}$		<0.005		0	0.09	<0.01	0.15	<0.14	0.008	0.11
$\bar{x}$±SEM				0	0.27 ±0.17	<0.01 ±0	<0.17 ±0.015	<0.22 ±0.08	0.02 ±0.16	0.13 ±0.02

[a]Serum antibody and Ig levels: mg/ml.

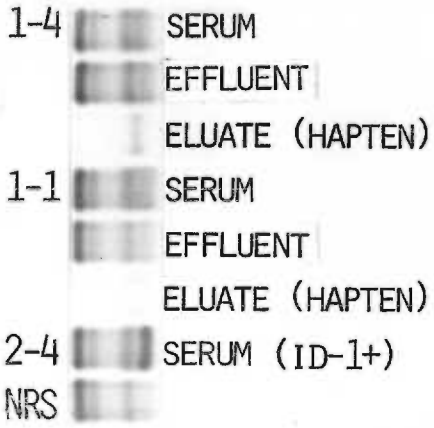
[b]Fractions eluted from N-AcGln 1A: mg/mg protein.

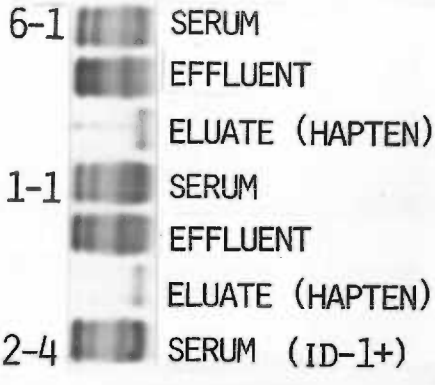
Figure 8. Cellulose acetate electrophoretic patterns of Id-1^{+} and Id-1^{-} sera and specifically purified antibody preparations from individual HPR rats.

CELLULOSE ACETATE ELECTROPHORETIC PATTERNS OF ID-1+ AND ID-1-

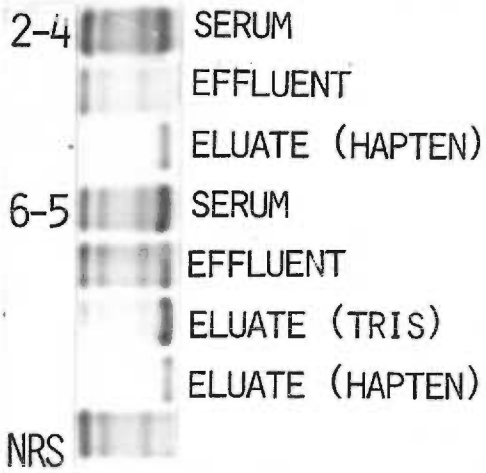
HPR RAT SERUM SAMPLES

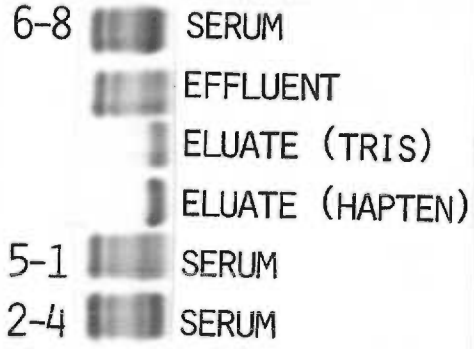
ID-1 NEGATIVE





ID-1 POSITIVE





which, in turn, allows one to gain a greater understanding of the extensiveness of the anti-SACHO immune repertoire.

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PAPER 1

Inheritance Patterns of Idiotype Expression: Maternal-Fetal Immune Regulatory Networks

Abstract

The production of ID-1, a cross-reactive idiotype associated with rat anti-group A streptococcal carbohydrate antibodies, by 11 strains of rats indicates that genes coding for Id-1 are in the germline. Its expression, however, follows a complex inheritance pattern. It was our intent in these studies to determine if immune responsiveness of streptococcus (GASV) immunized females could alter Id-1 expression of GASV-immunized progeny, and, in turn, introduce complications in Id-1 inheritance patterns. We observed that Id-1-specific immune reactivity of GASV-immunized females could induce significant alterations in Id-1 production by progeny. The relationship between maternal and progeny Id-1 was complex, reflecting the complexity of autologous regulation of Id-1 production, and could be the opposite of what one would predict based upon parental transfer of Id-1 regulatory genes. The non-genetic nature of the maternal regulatory influence was confirmed by foster-mother studies. We conclude that antigen-induced maternal immune responsiveness can exert a permanent regulatory influence on idiotype expression by progeny and consequently introduce error into the interpretations of idiotype inheritance patterns. The recognition of this maternal regulatory influence also lends further support to Jerne's hypothesis that idiotype-specific immune networks play a significant role in the regulation of immune responsiveness in vivo.

Introduction

Our laboratory has, for several years, studied the immune responsiveness of rats to group A streptococcal carbohydrate antigen (SACHO).

During this time we derived two rat strains of Sprague Dawley origin. One strain (HPR) was selectively inbred for high levels and the other (LPR), for low levels of precipitating anti-group A streptococcal carbohydrate (anti-SACHO) antibodies (Stankus and Leslie 1975). Owing to the high degree of molecular restriction of the anti-SACHO response we were able to identify and characterize a cross-reactive idiotype (Id-1) associated with rat anti-SACHO antibodies (Stankus and Leslie 1974, 1977, Klapper et al. 1976).

Briefly, Id-1 is a complex idiotype requiring both heavy and light chains for complete expression. It is expressed on from 0 to more than 90 percent of the anti-SACHO antibody molecules produced by 11 strains of rats, regardless of defined immunoglobulin allotypes or major histocompatibility haplotypes (Stankus and Leslie 1976a). IgG anti-SACHO antibodies are predominantly, but not exclusively, of the IgG_{2c} subclass, as is Id-1 (Leslie 1979). The association between Id-1 and IgG_{2c} is not absolute, however, since IgG_{2c}-positive, Id-1-negative anti-SACHO antibodies are produced (Leslie 1979). Recent studies identifying allotypic determinants on rat IgG_{2c} immunoglobulin molecules (G.A. Leslie, manuscript in preparation) indicate that Id-1 determinants are not linked exclusively with either of the IgG_{2c} alleles.

Although we observed a consistent pattern of inheritance for levels of precipitating anti-SACHO antibodies, when we later analyzed animals in these studies for idiotype expression, no simple pattern of inheritance was observed. Studies from other laboratories have shown that the maternal immune system can influence immune responsiveness of progeny (Dray 1962, Kindred and Roelants 1974, Ono et al. 1974, Stern

1976, Minami et al. 1977, Loor and Kelus 1978). All animals in our inheritance studies were immunized with group A streptococcal vaccine (GASV) prior to breeding. Therefore, it was possible that maternal idiotype-specific immune responsiveness had affected progeny Id-1 production and consequently altered the apparent patterns of Id-1 inheritance.

In this paper we will show that antigen-induced maternal immune responsiveness during the pre- and neonatal developmental periods can exert a permanent regulatory influence on idiotype expression by progeny. This observation lends further support to Jerne's hypothesis (Jerne 1974) that idiotype-specific immune networks play a significant role in the regulation of immune responsiveness in vivo. Also, since the maternal influence occurs in addition to and can alter the expression of progeny genes, one should use caution in the interpretation of inheritance patterns of structural and regulatory genes affecting idiotype production if females have been immunized prior to the birth of their progeny.

Materials and Methods

Animals. Inbred Fischer 344 (F344) rats, a strain giving a low response to SACHO, were purchased from Simonsen Laboratories, Gilroy, California. HPR rats, originally derived from a Sprague-Dawley colony maintained at Tulane University Medical School, have been selectively inbred by brother-sister matings in our laboratory for more than 7 years based upon the production of high levels of precipitating anti-SACHO antibodies (Stankus and Leslie 1975). The animals used in these

studies were from the F_{12-18} generation.

Immunization with group A streptococci. GASV was prepared from a lyophilized stock culture group A streptococcus, strain J17A4, originally obtained from Dr. Richard Krause and prepared as previously described (Stankus and Leslie 1975). To induce anti-SACHO antibodies, HPR and F344 rats were immunized at 3 months of age by intravenous (i.v.) or intraperitoneal (i.p.) injection, respectively, of 1 mg of GASV given three times per week on consecutive days for 2 to 3 weeks. Animals were bled 5 days after the last weekly injection.

Quantitation of anti-SACHO antibodies. Total anti-SACHO antibodies were assayed by radioimmunoassay (RIA) using a modified Farr technique (Bernstein et al. 1975). Purified SACHO, prepared as previously described (Stankus and Leslie 1975) was reacted with cyanogen bromide (CNBr) and coupled to tyramine. Specifically, 2.5 mg (dry weight) of SACHO was suspended in 5 ml of distilled water. Upon addition of 5 mg CNBr (Eastman: Rochester, New York) dissolved in 0.25 ml of distilled water, the mixture was quickly adjusted and maintained at pH 11 with 1 N sodium hydroxide until the pH stabilized. Tyramine (Sigma, St. Louis, Missouri), 10 mg dissolved in 2 ml of 0.5 N sodium bicarbonate buffer (pH 8.5), was subsequently added and the pH of the mixture was readjusted to 8.5. After overnight stirring at 4 C the preparation was dialyzed against 0.1 M phosphate buffer, pH 7.0, to remove unbound tyramine. Tyraminated SACHO was radiolabeled with 2.5 (m)Ci of ^{125}I (New England Nuclear, Boston, Massachusetts) using a chloramine T method (McConahey and Dixon 1966). To assay for anti-SACHO antibodies, dilutions of unknown serum samples were incubated

with ^{125}I-SACHO at 37 C for 1 h, then at 4 C overnight. The globulins were precipitated with 40% saturated ammonium sulfate $(NH_4)_2SO_4$ at 4 C and centrifuged in the cold. One-half of the 200 µl centrifuged mixture (supernatant) was removed and both this fraction and the remaining precipitate-containing fraction were counted in a Beckman Biogamma counter (Fullerton, California). The percent radiolabeled SACHO antibody is equal to:

$$\frac{\text{cpm in precipitate - cpm in supernatant}}{\text{cpm in precipitate + cpm in supernatant}} \times 100.$$

To determine the level of anti-SACHO antibody the percent SACHO bound by the unknown sample was compared with the percent SACHO bound by serial dilutions of a standard amount of anti-SACHO antibody. Each unknown serum sample was assayed in duplicate.

Levels of precipitating anti-SACHO antibodies were determined by reverse radial immunodiffusion (RRID) as has been previously described (Leslie and Hattier 1974).

Quantitation of Id-1. Id-1 was quantified by RIA using a heterologous anti-idiotypic antiserum (anti-Id-1) prepared in a rabbit against the $F(ab')_2$ fragment of specifically purified Id-1 positive anti-SACHO antibody molecules (Stankus and Leslie 1974, 1976b) obtained from one rat (5-1). The rabbit was injected subcutaneously at multiple sites with 500 µg of 5-1 $F(ab')_2$ mixed with an equal volume of Freund's complete adjuvant (FCA; Difco, Detroit, Michigan). Two weeks later the rabbit was given another 500 µg of the same immunogen mixture. A bleeding taken 4 weeks after the initial exposure to antigen gave positive reactions specifically with Id-1 preparations as determined by

Ouchterlony analysis (Leslie and Hattier 1974). The rabbit was subsequently bled at weekly or biweekly intervals for 6 months. During this time the rabbit was reimmunized periodically with 5-1 $F(ab')_2$ in Freund's incomplete adjuvant (FIA; Difco, Detroit, Michigan) to maintain high antibody titers. Antisera were analyzed by immunoelectrophoresis (IE) (Leslie and Clem 1969) and pooled based upon IE reactions and collection dates. The individual antisera pools were made Id-1 specific by passage over normal rat serum, normal rat IgG, and normal rat $F(ab')_2$ immunoadsorbent columns. The IgG fraction was obtained by precipitation with 40% saturated $(NH_4)_2SO_4$ prior to Sephadex G-200 gel filtration (Leslie and Clem 1969).

Id-1 was quantified by competitive inhibition RIA using the method previously described by Kindt and co-workers (1972). Specifically, 680 ng of anti-Id-1 dispensed in a 50 µl volume was added to unknown serum samples diluted in a 50 µl volume. Approximately 290 ng in a 50 µl volume of ^{125}I radiolabeled (McConahey and Dixon 1966) Id-1-positive anti-SACHO preparation (6-5 IgG) was added to the anti-Id-1 serum sample mixture which was then incubated at 37 C for 1 h. To precipitate the immune complex, 50 µl of a 1:4 dilution of sheep anti-rabbit IgG (PCL-RIA:lot 21-30-40-1, Portland, Oregon) was added to each tube. The use of radiolabeled Id-1 positive antibody molecules from a different individual rat (6-5 IgG) from the one used as immunogen in the rabbit (5-1 IgG) allowed for the more efficient detection and quantitation of the cross-reactive idiotype. Levels of Id-1 were quantified relative to a standard curve generated by dilutions of 6-5 IgG, which has more than 90 percent Id-1-positive

molecules. The sensitivity of the RIA relative to 6-5 IgG was 20 ng per sample. Unknown serum samples were assayed in duplicate and, to avoid interassay variations, all samples within a study were assayed at the same time. In this regard, we have observed that, although the percent inhibition produced by a particular sample remains constant in different assays, the absolute concentration of Id-1 will vary depending upon the specific activity of the ^{125}I radiolabeled Id-1 probe.

By RIA analysis, Id-1-anti-Id-1 interactions were approximately 50 percent hapten (N-acetyl-D-glucosamine; Sigma, St. Louis, Missouri) inhibitable. This indicates that some of the idiotypic determinants were associated with the antibody-combining site, whereas others, unique to anti-SACHO antibody molecules, were not intimately involved in antigen recognition. The immunodominant determinants of Id-1 are located on the heavy chains of the antibody molecule (unpublished observation), although, as indicated previously (Stankus and Leslie 1977), both heavy and light chains are required for complete expression.

In the pedigree diagram, animals were Id-1-positive if they produced Id-1 after either the primary or secondary series of immunizations as detected by Ouchterlony analysis (Stankus and Leslie 1974).

Neonatal treatments. Neonatal injections of GASV or SACHO were given i.p. within 24 h of birth. Neonates were fed 1 mg of GASV via intubation within 24 h of birth. To suppress Id-1 production in neonates, animals were injected i.p. on days 1 and 3 with either 50 µl of the gamma globulin fraction of purified rabbit anti-Id-1 serum or 100 µg of specifically purified rabbit anti-Id-1. More than 90 percent of these animals were suppressed for Id-1 synthesis (producing less

than 10 µg/ml of Id-1) following immunization with GASV at 3 months of age, regardless of the anti-Id-1 preparations used. Control animals, neonatally injected with normal rabbit gamma globulin or normal rabbit IgG, were not suppressed for Id-1 production.

Id-1 suppression of adult animals. To suppress Id-1 production in adult rats, at 3 months of age animals were injected i.p. with 200 µg of specifically purified rabbit anti-Id-1. The injection was repeated 2 days later, and on day 5 the GASV immunization protocol was initiated. Control animals received 200 µg injections of normal rabbit IgG in place of the specifically purified anti-Id-1.

Induction of autologous anti-Id-1 antibodies. To induce autologous anti-Id-1 immunity, F344 and HPR rats were immunized with the $F(ab')_2$ fragment and the whole IgG molecule, respectively, of specifically purified Id-1-positive anti-SACHO antibodies of restricted heterogeneity, prepared as previously described (Stankus and Leslie 1974, 1976b, Leslie and Hattier 1974). Rats were injected initially with 100 µg of Id-1-positive anti-SACHO in FCA. A total of 500 to 600 µg was injected into each rat over a 4 to 5 month period. Later injections of Id-1 were given in FIA or saline. Induction of autologous anti-Id-1 antibodies was monitored by Ouchterlony analysis (Leslie and Hattier 1974).

Statistical analysis. Where indicated, results were statistically analyzed with a directional, two-sample, t-statistical evaluation test.

Results

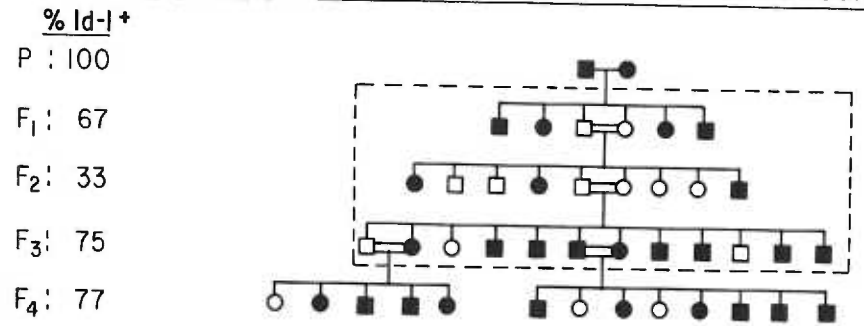
Genetic pattern of inheritance of Id-1 expression. When animals involved in our previous studies on the inheritance of high or low production of anti-SACHO antibodies were monitored for idiotype, it was evident that Id-1 expression involved complex multigene interactions. As shown in Figure 1, sequential breeding of either Id-1-negative (upper pedigree) or Id-1 positive rats (lower pedigree) did not produce Id-1-positive progeny in proportions in agreement with Mendelian patterns of inheritance of a single gene. Regardless of whether animals were of the high or low responding strain, idiotype expression by progeny appeared to increase in later generations, independent of its expression or lack of expression by the parents.

Relationship between maternal concentrations of Id-1 and subsequent Id-1 production by progeny. In previous studies we observed natural heterogeneity in the ability of animals within each litter and strain to produce Id-1. As shown in Figure 2, there is considerable variation in the concentrations of Id-1 produced by siblings of nonimmune F344 parents. Mean levels of Id-1 produced by the different litters remained very constant, however. This indicates that animals which have minimal genetic differences still maintain individually specific mechanisms for regulating Id-1 production.

We next immunized F344 females with GASV and compared the levels of Id-1 produced by these females at the time of birth of progeny, with Id-1 production of progeny following GASV immunization at 3 months of age. To eliminate possible influences of paternal immune responsiveness, females were mated with nonimmune F344 males. It is apparent

Figure 1. Id-1 expression by rats selectively inbred for the production of high or low concentrations of precipitating anti-SACHO antibodies. (■ , ● = Id-1 positive ♂ and ♀ , respectively; □ , ○ = Id-1-negative ♂ and ♀ , respectively. Mean parental anti-SACHO concentrations: (1) High responders, P=2.7, F_1=1.8, F_2=6.7, F_3=20 mg/ml. (2) Low responders, P=0.4, F_1=0.5, F_2=0.3, F_3=0.7, F_4=0 mg/ml.

Inbred for High Levels of Anti-SACHO Antibodies



Inbred for Low Levels of Anti-SACHO Antibodies

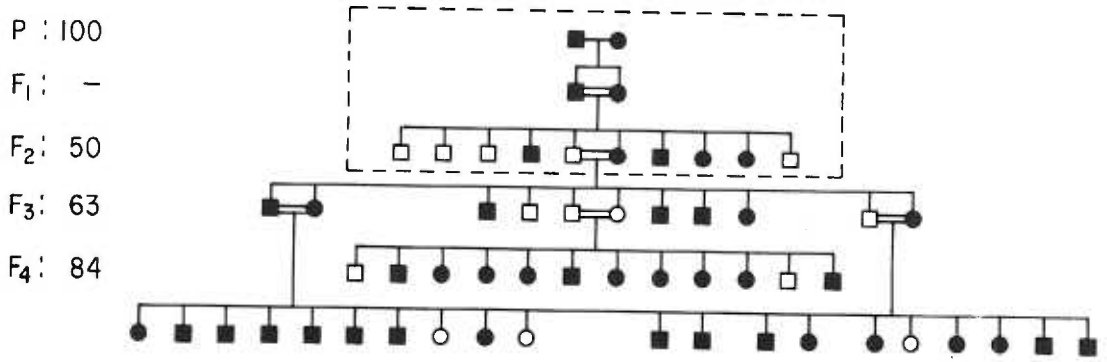
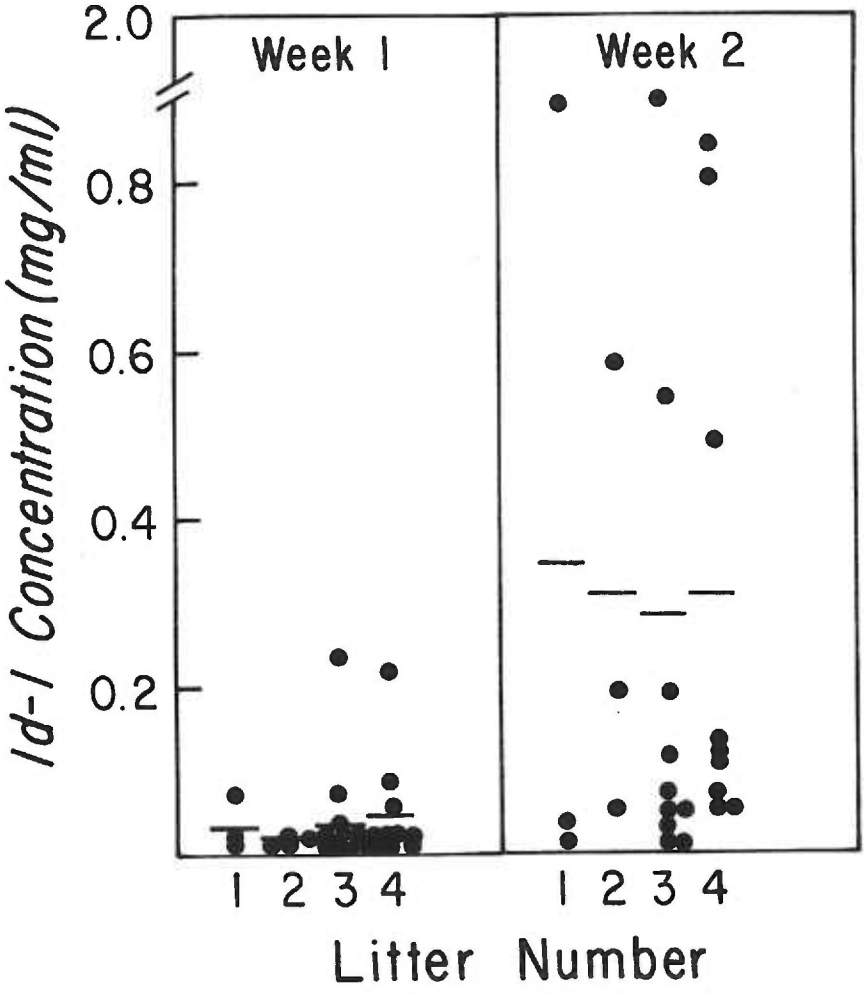


Figure 2. Concentration of Id-1 produced by F344 progeny of non-GASV-immune females after 1 and 2 weeks of GASV immunization. Bars represent mean levels of Id-1 produced by each litter.

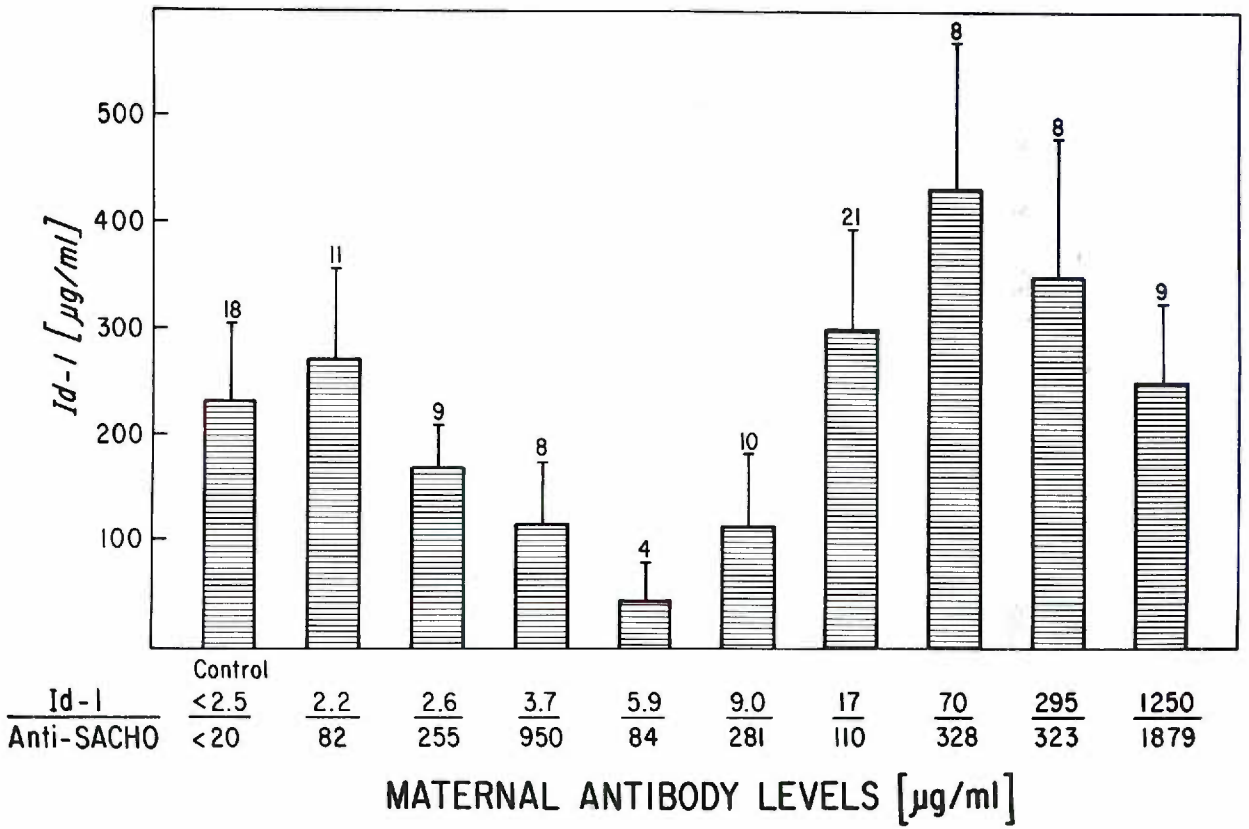

Id-1 Concentration (mg/ml)
2.0
0.8
0.6
0.4
0.2
Week 1
Week 2
1 2 3 4
1 2 3 4
Litter Number

from Figure 3 that the potential of offspring to produce Id-1 was altered, and that the extent of the alteration appeared to be related to maternal Id-1 concentrations. Although not shown, fluctuations in progeny Id-1 production were not consistently paralleled by alterations in anti-SACHO antibodies, indicating that Id-1 production could be regulated independently of anti-SACHO production.

These results provided us with the first evidence suggesting that GASV-induced maternal Id-1-specific immune reactivity could permanently influence the potential of progeny to produce Id-1. The biphasic relationship between maternal and progeny Id-1 production, however, emphasized the complexity of this relationship.

As shown in Figure 2, F344 rats produce from 0 to more than 1500 µg/ml of Id-1 following 2 weeks of GASV immunization. F344 rats producing less than 10 µg/ml of Id-1 throughout the hyperimmunization protocol we define as being Id-1-suppressed. Based upon this definition, five mothers in Figure 3 are Id-1 suppressed, with two mothers having borderline suppression. Yet, it is apparent that suppressed Id-1 production in mothers may or may not be associated with low Id-1 production in progeny (i.e., progeny of the female producing 5.9 µg/ml vs. progeny of the female producing 2.2 µg/ml of Id-1). Similarly, offspring from the female producing the highest Id-1 concentration (1250 µg/ml) produced lower levels of Id-1 than did offspring of females having one-tenth the amount of idiotype. The discrepancies between these results make direct interpretations of the relationship between maternal and progeny Id-1 production impossible. Further

Figure 3. Mean ± standard error of the mean ($\bar{x}$ ±SEM) of Id-1 levels produced by GASV-immunized F344 progeny related to increasing maternal Id-1 levels. Represented on the abscissa are maternal Id-1 and anti-SACHO levels at parturition as extrapolated from levels of Id-1 and anti-SACHO produced by female rats from 2-6 weeks prior to and after birth of offspring. The number above the bar represents the number of offspring per litter from each of the respective females with the exception of offspring from females having 17 μg/ml of Id-1 which represents a pool of two litters. Control offspring are a pool from three nonimmune rats. Progeny Id-1 levels after 2 weeks of immunization are represented relative to the ordinate axis.


Id-1 [µg/ml]
500
400
300
200
100
18
11
9
8
4
10
21
8
8
9
Control
Id-1 <2.5 2.2 2.6 3.7 5.9 9.0 17 70 295 1250
Anti-SACHO <20 82 255 950 84 281 110 328 323 1879
MATERNAL ANTIBODY LEVELS [µg/ml]

studies, however, designed to help clarify this relationship (discussed below) have indicated that the history of the female prior to GASV immunization (for example, the mechanism for inducing Id-1 suppression) has a very important influence on determining the effect maternal immune responsiveness has on progeny Id-1 production. For this reason it is important to note that in these initial studies, although all mothers had been hyperimmunized with GASV, they represent a composite of females of different backgrounds, selected for this study solely on the basis of their ability, or lack of ability to produce Id-1. In addition, none of the females had been specifically suppressed for Id-1 production.

We have recently completed a comprehensive study comparing the potential of mothers to produce Id-1 with subsequent Id-1 production by their progeny. In these studies we observed that if HPR females had been suppressed for Id-1 production during neonatal development and were mated with either nonimmune or GASV-immune males, their progeny produced significantly higher concentrations of Id-1 ($p<0.05$) than did control progeny (Table 1). The Id-1 to anti-SACHO antibody ratio of these progeny was also consistently different from that of control progeny. In contrast, progeny of females naturally producing low Id-1 levels (rabbit IgG injected female in experiment 1, and the control female in experiment 2) produced low Id-1 concentrations. Previously we have observed that approximately 10 percent of the GASV-immunized HPR rats naturally lack Id-1 production. The lack of Id-1 production by these control females was most likely a reflection of this natural lack of Id-1

Table 1. Id-1 production by progeny of GASV-immunized females producing low concentrations of Id-1

Parents					Progeny		
Experiment	Treatment		Id-1[a]	Anti-SACHO	n	Id-1[b]	Anti-SACHO
1	HPR F_{18}	♀ anti-Id-1 neonatally	0	4100			
		GASV immune[c] (n=2)			18	1702± 332	1583± 275
		♂ nonimmune	-	-			
		♀ nonimmune (n=2)	-	-			
		♂ nonimmune	-	-	9	733± 224	4026± 713
		♀ rabbit IgG neonatally	0	1200			
		GASV immune[c] (n=1)			8	383± 183	2769± 838
		♂ nonimmune	-	-			
2	HPR F_{15}	♀ maternally transferred	9	9500			
		Id-1 suppression[d] (n=1)					
		GASV immune[e]			10	3652±1134	2921± 807
		♂ GASV immune[f]	70	4910			

Table 1. (Continued)

Parents				Progeny		
Experiment	Treatment	Id-1[a]	Anti-SACHO	n	Id-1[b]	Anti-SACHO
	♀ control females (n=2)	3350	4574			
	♀ GASV immune[e]			22	1717± 682	4226± 802
	♂ GASV immune	70	4910			
	♀ control female (n=1)[g]	16	4380			
	♀ GASV immune[e]			8	370± 235	7551±1931
	♂ GASV immune	70	4910			

[a]Parental Id-1 and anti-SACHO concentration (μg/ml) following 3 week GASV immunization protocol. Id-1 quantified by RIA, precipitating anti-SACHO by RRID. When more than one animal is involved, mean antibody concentrations are indicated.

[b]Due to interassay variations, comparisons of Id-1 production by animals within different experiments are not valid. All animals within an experiment were assayed in the same Id-1 RIA. Mean±SEM are indicated.

Table 1. Footnotes (Continued)

[c]Progeny of GASV-immune females were born within a 1 week period.

[d]Mothers in this study were progeny of Id-1 immune females and were permanently suppressed for Id-1 production.

[e]Progeny of GASV-immune females were born within a 2 week period.

[f]All females within this experiment were allowed to breed with the same group of three males.

[g]Control females were progeny of normal rat IgG-immune females. Of the 11 progeny, two produced suppressed concentrations of Id-1.

production, rather than being related to their treatment, since none of the other control animals in the same study were Id-1 suppressed.

We have also observed that progeny of two HPR females, suppressed for Id-1 synthesis as adults by the injection of anti-Id-1 just prior to the initiation of GASV-immunization, did not have enhanced Id-1 production. In fact, 8 of the 13 progeny were suppressed for Id-1 synthesis, compared with 1 of 10 suppressed progeny from control females. The mean Id-1 concentration of control progeny was 1156 μg/ml. Like neonatally induced suppression, Id-1 production by animals receiving anti-Id-1 as adults was permanently suppressed as assessed by its maintenance following periodic rechallenges with GASV over a 6 month period.

These studies indicate that low Id-1 production by females at the time of birth of their progeny per se cannot be directly related to enhanced or suppressed Id-1 production by progeny. There does, however, appear to be a relationship between the mechanism by which Id-1 suppression was induced in females and their ability to alter the potential of their progeny to produce Id-1. Using this criterion, at least three functionally distinct mechanisms for maintaining Id-1 suppression can be recognized: (1) suppression which appears to require neonatal induction and is recognized by transfer of enhanced Id-1 production to progeny. (Preliminary evidence indicates that GASV-immunization of females is a prerequisite for the transfer of this influence to progeny.) (2) Id-1-specific suppression, acquired as an adult, which induces Id-1 suppression in the majority of progeny. (3) The natural acquisition of Id-1 suppression which occurs in approximately 10 percent of the HPR

rats and may result from the transfer of a specific combination of regulatory genes favoring low Id-1 production.

In the same study we also compared Id-1 production by progeny of parents or females known to produce very high or median Id-1 concentrations. As represented in Table 2, progeny of females having the potential to produce extremely high levels of Id-1 had a tendency themselves to have significantly lower Id-1 production levels ($p<0.05$) than did progeny of nonimmune females. These results are in contrast to those expected if gene transfer was the sole factor affecting Id-1 production and suggest that Id-1-specific immunity is induced in the high Id-1 producing females and is recognized by its significant influence on Id-1 production by progeny. As shown in experiment 3, progeny of females producing median concentrations of Id-1 did not produce significantly different Id-1 levels from control progeny. Similarly, in an additional study we observed that progeny of three females producing approximately the same concentrations of Id-1 (688±99 μg/ml) also produced approximately the same median concentrations of Id-1 (1513±47 μg/ml). These later studies suggest that: (1) only females producing very high Id-1 levels could transfer significant Id-1-specific immunity to progeny during this period after GASV-immunization, and (2) maternal transfer of antigen is not the dominant influence affecting Id-1 production by progeny. If transfer of GASV or processed GASV was responsible for the maternal influence, one would expect all progeny of the GASV-immune females within the three experiments to have comparable alterations in their Id-1 production relative to controls. However, in experiment 3, contrary to

Table 2. Comparison of Id-1 production by GASV-immunized rats to subsequent Id-1 production by GASV-immunized progeny

Parents					Progeny		
Experiment	Treatment		Id-1[a]	Anti-SACHO	n	Id-1[b]	Anti-SACHO
1	HPR F_{12}	♀GASV immune[c]	17200	18000	7	356±145	509± 97
		♂GASV immune	13530	30133			
		♀nonimmune	-	-	3	760±218	708± 74
		♂nonimmune	-	-			
2	HPR F_{15}	♀GASV immune	13500	12850	5	200±126	160±100
		♂GASV immune	n.d.[d]				
		♀nonimmune	-	-	7	1098±383	1340±220
		♂nonimmune	-	-			
3	HPR F_{13}	♀GASV immune	2100	1700	10	992±307	401± 93
		♂GASV immune	8500	9900			
		♀nonimmune	-	-	7	696±358	513±103
		♂nonimmune	-	-			

[a]Parental and progeny Id-1 and anti-SACHO were assayed as in Table 1.

[b]Progeny antibody concentrations ($\bar{x}$±SEM) after 1 week of GASV immunization are represented.

Table 2. Footnotes (Continued)

[c]Immune females were given their last injection of GASV 53 days (experiment 1), 71 days (experiment 2) and 76 days (experiment 3) prior to birth of their progeny.

[d]n.d. indicates not determined.

experiments 1 and 2, progeny of the GASV-immune female have, if anything, a tendency for enhanced Id-1 production compared with control progeny.

Fostering of offspring by females producing high and low Id-1 levels. Since the major transmission of maternal rat immunoglobulins occurs during nursing between 3 and 21 days of age (Halliday 1955, Brambell 1970, G.A. Leslie, manuscript in preparation), it is conceivable that alterations in progeny Id-1 expression, induced by maternal immune reactivity, could be transferred during nursing. In this regard, we observed Id-1 to be transferred from GASV-immune females to progeny, reaching maximum levels in the neonatal circulation approximately 15 days after birth (Figure 4).

To examine the possibility that immune responsiveness of foster mothers could alter the potential of offspring to express Id-1, two separate studies were performed. In the first, we bred GASV-immunized F344 females either neonatally suppressed for Id-1 production or producing very high levels of Id-1. At birth the litters were divided and one-half of the litter remained with the true mother, while the other half was fostered by a female having the opposite Id-1-producing potential. As shown in Table 3 (experiment 1), when progeny of females producing high levels of Id-1 were fostered by neonatally Id-1 suppressed females, the transferred progeny produced significantly higher concentrations of Id-1 ($p<0.025$) than did those remaining with their high-Id-1-producing mother. The enhanced Id-1 production by progeny from neonatally Id-1-suppressed females as compared with that of progeny of females producing high levels of Id-1 ($p<0.025$), and the

Figure 4. Id-1 level of a GASV-immunized HPR female rat (▲-----▲) prior to and after parturition, and her six non-immune progeny (●——— ●; $\bar{x}$±SEM) during the neonatal development period. An open square represents the mean Id-1 level of 23 progeny from two GASV-immunized females lacking detectable Id-1.

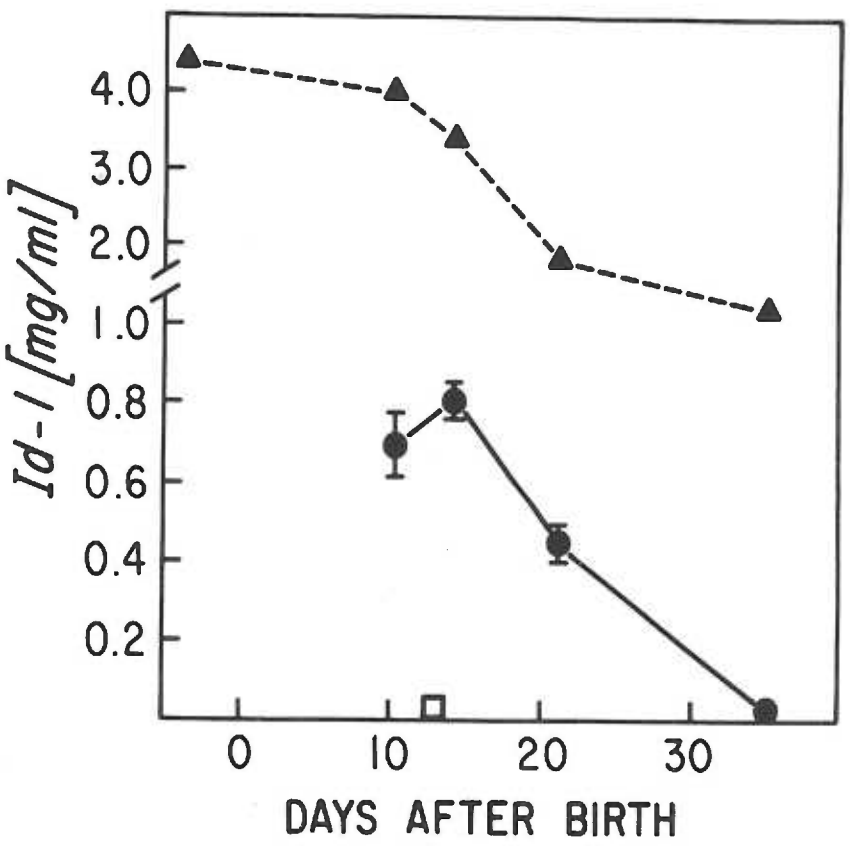

4.0
3.0
2.0
1.0
0.8
0.6
0.4
0.2
Id-1 [mg/ml]
0
10
20
30
DAYS AFTER BIRTH

Table 3. Id-production by GASV-immunized F344 progeny fostered by either neonatally Id-1 suppressed or high-Id-1 producing females

	Maternal[a]	Foster mother		Progeny[b]	
	Id-1	Id-1	n	Id-1	Anti-SACHO
Experiment 1	a) >750 (n=2)	>750 (n=2)	9	419±142	473± 90
	>750 (n=2)	<3 (n=2)	6	1144±269	597±106
	b) <3 (n=2)	<3 (n=2)	6	1249±359	547±145
	<3 (n=2)	>750 (n=1)	6	799±358	498± 66
Experiment 2	a) nonimmune (n=2)	nonimmune (n=2)	9	474±183	332± 69
	nonimmune (n=2)	<1 (n=1)	4	1034±446	598± 91
	b) nonimmune (n=2)	nonimmune (n=2)	8	534±178	443±102
	nonimmune (n=2)	$\bar{x}$500 (n=2)	8	111±60	388± 61
Experiment 3	nonimmune (n=2)	nonimmune (n=2)	9	360±145	288± 56
	nonimmune (n=2)	$\bar{x}$220 (n=2)	10	588±139	430± 67

Table 3. Footnotes

[a]Represented are maternal and foster mother concentrations of Id-1 present at time of birth of progeny. Immune females were given their last injection of GASV a mean of 42 days (experiment 1a), 42 days (experiment 1b), 51 days (experiment 2a), 47 days (experiment 2b), and 75 days (experiment 3) prior to birth of their progeny.

[b]Id-1 quantified by RIA. Levels of precipitating anti-SACHO antibodies quantified by RRID. Mean±SEM (μg/ml) are represented.

ability to transfer this influence to progeny of such females is consistent with our previous results (above). If progeny of neonatally suppressed females, however, were nursed by females producing high Id-1 concentrations, the tendency for enhanced Id-1 production was lost.

In the second study, to dissect more carefully the contributing influence of the neonatally Id-1-suppressed female from that of the high-Id-1-producing female, each of these females was allowed to foster progeny of nonimmune females. It is apparent from Table 3 (experiment 2) that the neonatally Id-1-suppressed females and the high-Id-1-producing females could enhance and suppress ($p<0.025$) idiotype production, respectively, by progeny of the nonimmune females which they fostered. As might be expected there was no significant alteration of Id-1 production by progeny of high-Id-1-producing or neonatally Id-1-suppressed females fostered on nonimmune females as compared with that of their littermates remaining with their true mother. As shown in experiment 3 (Table 3), females having lower Id-1 concentrations (220 μg/ml) did not significantly influence the Id-1-producing potential of the progeny they fostered, emphasizing that transferable Id-1-specific immunity, under the conditions of this study, required the production of high Id-1 concentrations by females.

These studies confirm that nongenetic influences of GASV-immunized females can alter the potential of progeny to express Id-1. It is also apparent that the maternal influence can obscure patterns of genetically controlled Id-1 expression, since the influence of these studies could be the opposite of that which one would expect from the inheritance of

maternal Id-1 structural and regulatory genes. It is also evident that maternal alterations in Id-1 production can occur independently of concomitant alterations in the production of precipitating anti-SACHO antibodies.

The effect of neonatal exposure to antigen on subsequent Id-1 production. The above studies strongly argue that idiotype-specific immune reactivity of GASV-immunized females altered the potential of progeny to produce Id-1. There remains the possibility, however, that transfer of maternally derived antigen from the immune females could also directly affect Id-1 production by progeny. As previously mentioned, Tables 1 and 2 indicate that litters born at approximately the same time to females immunized with GASV at the same time differ significantly in their potential to produce Id-1. For example, in Table 1 (experiment 1) litters of GASV-immunized females produced significantly different levels of Id-1, although all progeny within the experiment should have had the same opportunity to acquire antigen from their mothers. A similar lack of correlation between the time of last injection of antigen to females and subsequent Id-1 production by progeny is observed in both Table 3 and progeny represented in Figure 3. It therefore appears that if maternally transferred antigen induces alterations in progeny Id-1 production, it either induces inconsistent effects or its influence on Id-1 production by progeny is dominated by the influence induced by maternal transfer of idiotype-specific immunity.

In order to further understand the influence neonatal exposure to antigen can have upon an animal's subsequent Id-1 production, we both

fed and injected antigen into neonatal rats. Eight different strains of rats, either inbred, F_1, or F_2 crosses, were injected i.p. neonatally with 50 μg of GASV. In addition, HPR rats were injected neonatally with comparable amounts of the purified carbohydrate (SACHO), and F344 rats were fed 1 mg of GASV neonatally. Unlike what might have been expected, neonatal exposure to this relatively high concentration of antigen did not induce specific tolerance when the animals were challenged with GASV as adults. In contrast, all progeny fed GASV and three of the eight groups injected with GASV neonatally had significantly enhanced production ($p<0.05$) of anti-SACHO antibodies compared with control animals. Although Id-1 production in these later studies tended to be enhanced, there still was no significant difference between the animals neonatally exposed to GASV and control animals.

One can conclude, at least from these experiments, that neonatal exposure to GASV can significantly enhance the later ability of animals to respond to challenges with GASV. The influences of neonatal exposure to antigen on Id-1 production, however, appear to be secondary to those on anti-SACHO and are not significant. Similarly, previous studies supporting the significant relationship between maternal and progeny Id-1 production suggest that if maternal transfer of antigen has a significant effect on Id-1 production by progeny, this takes a secondary role to influences induced by the transfer of maternal idiotype-specific immune reactivity.

The influence of maternal Id-1-specific immune reactivity on progeny Id-1 expression. Although we have indicated that maternal Id-1 can be transferred to progeny, eight studies in which Id-1 was injected i.p.

into neonatal rats indicated that neonatal exposure to Id-1 per se was inefficient in altering the potential of the individual to produce Id-1 (Olson and Leslie manuscript in preparation). To determine if maternal Id-1-specific immune reactivity could influence progeny Id-1 production independently of maternal transfer of antigen, we selectively manipulated idiotype-specific immunity of females, and determined if such manipulations could alter idiotype expression by progeny. For this study F344 and HPR females were extensively immunized with the $F(ab')_2$ fragment and the whole IgG molecule, respectively, of Id-1-positive anti-SACHO. After 4 to 5 months of immunization, Ouchterlony analysis revealed that some of the females produced antibodies that precipitated with Id-1-positive but not Id-1-negative anti-SACHO antibodies. All females were then mated and their offspring immunized with GASV at 3 months of age. Progeny Id-1 and anti-SACHO production were quantified and compared with that of progeny of nonimmune and normal IgG-immune female rats. As shown in Figure 5, progeny of F344 females immunized with Id-1 produced significantly lower levels of Id-1 ($p<0.05$) than did progeny of nonimmune females. Similarly, as shown in Figure 6, progeny of Id-1-immunized HPR females had significantly suppressed Id-1 production (p 0.005) compared with progeny of either nonimmune or normal IgG-immune female rats. In both studies, Id-1 suppression was not associated with suppression of anti-SACHO antibodies.

It can be concluded that maternal Id-1 specific immune reactivity has the potential to selectively alter Id-1 production of GASV-immunized progeny without significantly altering anti-SACHO production. Interestingly, extensive immunization of females with Id-1 induces an immune

Figure 5. Id-1 and anti-SACHO levels ($\bar{x}\pm$SEM) of GASV-immunized offspring of Id-1 immunized and nonimmune F344 females. Progeny Id-1 and anti-SACHO production after 1 and 2 weeks of GASV immunization are represented. The p value of <0.05 was calculated by t-test analysis. Numbers above the bar indicate the number of progeny per group.

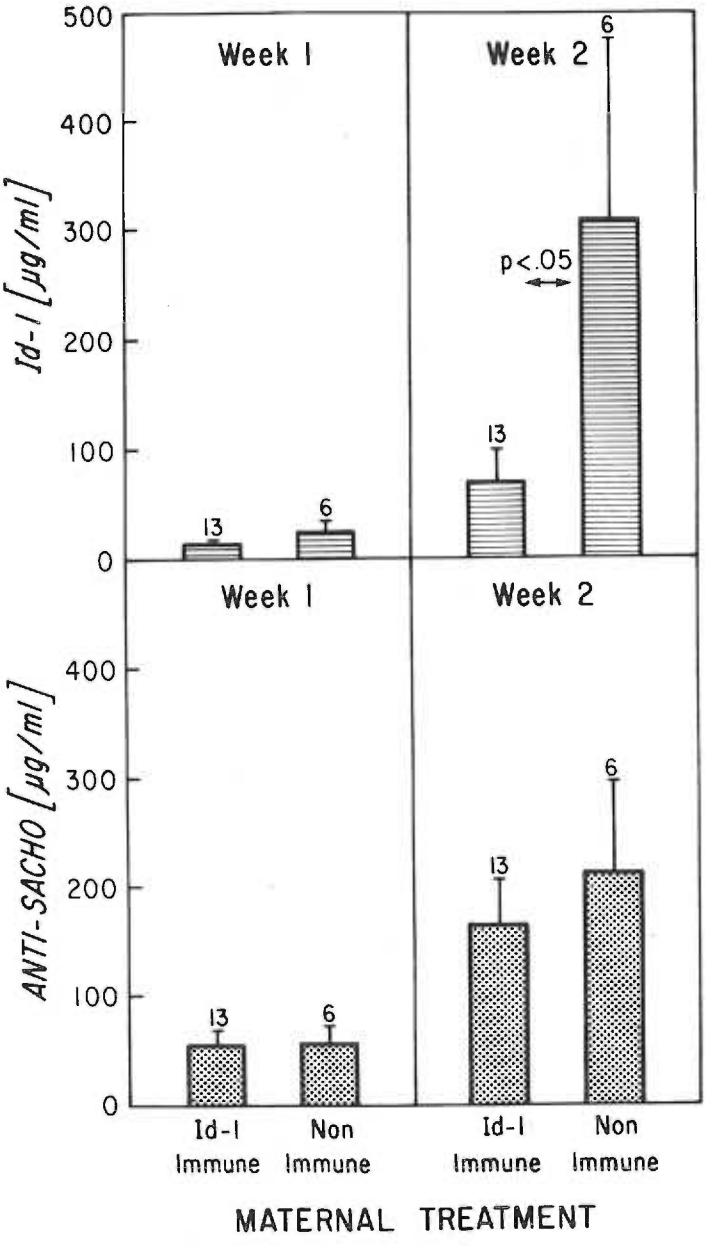
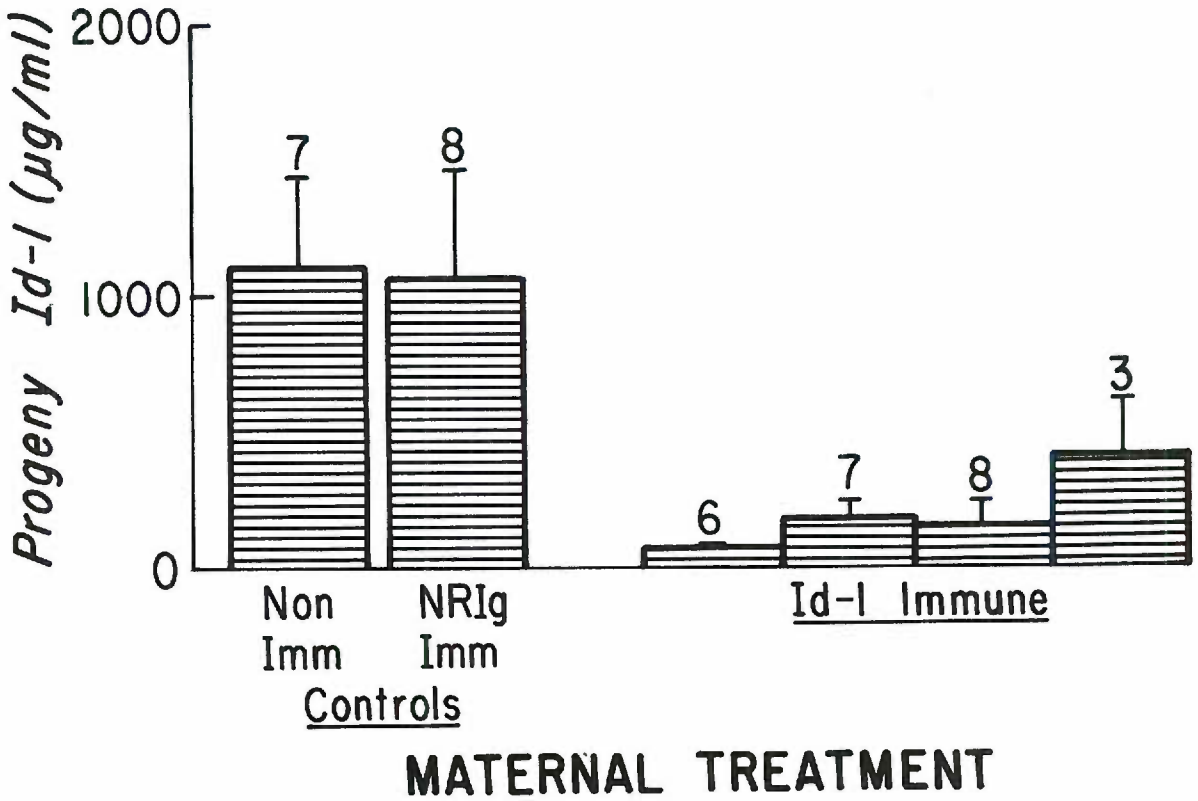

Week 1
Week 2
Id-1 [μg/ml]
500
400
300
200
100
0
p<.05
13
6
13
6
Week 1
Week 2
ANTI-SACHO [μg/ml]
400
300
200
100
0
13
6
13
6
Id-1 Immune
Non Immune
Id-1 Immune
Non Immune
MATERNAL TREATMENT

Figure 6. Id-1 and anti-SACHO levels ($\bar{x}\pm$SEM) of GASV-immunized HPR offspring of Id-1-immunized, normal IgG-immunized and non-immune female rats. Progeny Id-1 concentrations after 1 week of GASV immunization are represented. Id-1 production by progeny of Id-1-immune vs those of non-Id-1-immune females was significantly different ($p<0.005$).


Progeny Id-1 (µg/ml)
2000
1000
0
7
8
6
7
8
3
Non Imm
NRIg Imm
Controls
Id-1 Immune
MATERNAL TREATMENT

response which transfers Id-1-specific suppression to progeny, just as females producing the highest concentrations of Id-1 transfer suppressed Id-1 production to progeny. This may implicate the involvement of similar mechanisms (i.e., Id-1 induction of auto-Id-1 reactivity) for the transfer of Id-1 suppression to progeny. The fact that animals suppressed for Id-1 synthesis as adults by the injection of heterologous anti-Id-1 also transfer Id-1 suppression to progeny may be indicative of the involvement of autologous anti-Id-1 antibodies in the induction of this suppression.

Discussion

The purpose of these studies was to increase our understanding of the mechanisms regulating idiotype suppression which, in turn, might help explain the complex inheritance patterns of Id-1. Previously we found Id-1 to be expressed by 11 strains of rats, indicating that the Id-1 structural gene was in the germline. However, Id-1 expression did not follow Mendelian patterns of inheritance for a single gene. Although it was evident that Id-1 expression involved multigene interactions, in our inheritance studies we did not take into consideration that immune reactivity of GASV-immunized parents might be further complicating patterns of Id-1 expression. Since it was apparent from studies in other laboratories that the maternal immune system could influence progeny immune responsiveness, we chose to examine whether maternal immune reactivity at the time of birth of progeny could influence the potential or progeny to produce Id-1.

The studies presented in this paper show that immunization of

females with GASV prior to the birth of their progeny could alter the potential of progeny to express Id-1. Surprisingly, maternal immune reactivity could induce influences on progeny Id-1 production the opposite of what would have been predicted based upon the transfer of maternal regulatory genes. That is, females producing very high concentrations of Id-1 transferred suppressed Id-1 production to progeny, and females neonatally suppressed for Id-1 transferred enhanced Id-1 production to progeny. Foster mother studies confirmed the regulatory influence to be of maternal origin. The phenotypic expression of Id-1 is therefore, first, affected by the inheritance of structural and regulatory genes required for Id-1 production and, second, by external regulatory influences, in this case maternal immune responsiveness. As is evident from these studies, the maternal influence has the potential to obscure inheritance patterns for idiotype expression. Consequently, one should use caution in interpreting inheritance patterns of structural and regulatory genes affecting idiotype production if females have been immunized prior to the birth of their progeny.

The maternal influence on progeny Id-1 production has also served as a means of identifying different functional mechanisms of suppression in vivo. These mechanisms include (1) suppression which induces enhanced Id-1 production in progeny, (2) suppression associated with transfer of Id-1 suppression to progeny, and (3) suppression which most likely results from the transfer of genes favoring low Id-1 production. As idiotypic systems used by other laboratories have become more precisely defined, it is increasingly evident that many functionally

different lymphocytes have the same cell-surface receptor (the idiotype) (Hetzelberger and Eichman 1978) and that cells responsible for the same effect (i.e., the induction of idiotype suppression) have different cell receptors, idiotype or anti-idiotype (Sy et al. 1980, Weinberger et al. 1980). Similarly, functionally different mechanisms or cell types associated with Id-1 suppression can now be recognized from studies on the transfer of idiotype-specific influences from mother to progeny. The identification and a complete understanding of the in vivo potential of these mechanisms is an essential correlate to the identification of idiotype-specific cell types and their function in vitro.

Since our study was designed to examine the influence of antigen-induced idiotype-specific immunity on Id-1 production by progeny, experimental females were immunized with GASV before mating. Therefore, the possibility remains that maternally transferred antigen could also be directly altering Id-1 production. Although such an influence is difficult to eliminate, the maternal idiotype-specific immunity appears to dominate in the studies we have described for the following reasons: (1) there was no sigificant relationship between GASV immunization of females and subsequent Id-1 production by progeny, nor between the time of the last injection of antigen into female rats and progeny Id-1 production; (2) there was, however, a significant relationship between GASV-induced maternal Id-1-immune reactivity and subsequent Id-1 production by progeny; and (3) direct exposure of neonates to GASV or SACHO, either via intubation feeding or by i.p. injection, induced no significant alteration in the animal's subsequent production of Id-1, even

though in some studies significant enhancement of anti-SACHO production was induced. We also know that idiotype-specific maternal immune reactivity can influence progeny Id-1 expression and that neonatal injection of anti-Id-1 or cells from Id-1-immune or Id-1-suppressed females can significantly alter the potential to express Id-1 (Olson and Leslie manuscript in preparation).

Previous studies indicated that regulation of Id-1 synthesis was independent of regulation of anti-SACHO production. Similarly, maternally induced influences on Id-1 production were not consistently paralleled by alterations in anti-SACHO concentrations. These results differ from those obtained in studies of maternal transfer of idiotype suppression to progeny by Weiler and co-workers (1977) on mouse responsiveness to α(1-3)dextran and by Cosenza and co-workers (1977) on mouse responsiveness to phosphorylcholine. It was observed by both groups that maternally induced idiotype suppression in progeny was consistently associated with concomitant suppression of total antibody production to the respective antigen.

Steele (1979) has recently reexamined the role of "somatic selection and adaptive evolution" in influencing an individual's immune potential. The significance of this influence was confirmed by transfer of H-2-specific tolerance from father to offspring (Gorczynski and Steele 1980). In our most recent studies we have eliminated paternal influences by using nonimmunized males. We are currently examining the possibility that additional alterations in progeny Id-1 and anti-SACHO production may be induced by acquired alterations in germline DNA.

Based upon our own studies and on data from other laboratories, we postulate the existence of significant maternal-fetal regulatory immune networks. One means of recognizing such networks is to study the relationships between maternal and progeny idiotype expression. The maternal-fetal immune network would consist of antibodies or immune reactive cells transiently present in mothers during gestation and/or the neonatal developmental period which would have the ability to permanently alter the expression of the immune repertoire of progeny. In this regard, it is well established in humans that maternal antibodies and lymphocytes are transplacentally transferred and can affect the neonate (Loke 1978). However, these maternal influences have generally been considered transient, lasting until the maternal factor or cell has been eliminated. Studies in animals systems (Dray 1962, Kindred and Roelants 1974, Ono et al. 1974, Stern 1976, Minami et al. 1977, Loor and Kelus 1978, Weiler et al. 1977, Auerbach and Clark 1975, Beer et al. 1977, Jarrett and Hall 1979) and some studies in humans (Peer 1958, Field and Caspary 1971, Barnetson et al. 1976, Evans et al. 1976) provide evidence that the maternal immune system can permanently affect immune responsiveness of offspring. Therefore, maternally induced influences (via idiotype-anti-idiotype, cell-anti-cell receptor, or anti-self interactions) could result in a specific enhancement or suppression of immune reactive clones in progeny. As is emphasized by our studies, antigen-induced idiotype-specific immunity can be maternally transferred and can subsequently alter Id-1 production by progeny, independent of specific manipulation of the mother's immune repertoire with

idiotype or anti-idiotype probes. The recognition of antigen induction of such regulatory influences in humans could provide a means of explaining the acquisition by certain individuals of, for example, hypersensitivities or autoimmunities which appear to be inherited but in an unpredictable pattern.

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PAPER 2

Mechanisms of maternal immunological influences on progeny idiotype production

Abstract

We previously showed that antigen-induced, idiotype-specific immune reactivity of female rats had the potential to permanently alter the ability of progeny to produce the same idiotype. The idiotype used to monitor these influences was Id-1, a cross-reactive idiotype associated with rat anti-Group A streptococcal carbohydrate antibodies. To increase our understanding of the mechanisms associated with these maternal influences, we compared maternally induced alterations in progeny Id-1 production to alterations induced by the administration of neonatal rats with Id-1 related immune components.

Females, which maintained the same Id-1 suppressed state, but differed in the age at which suppression was induced (neonatal vs. adult induction), exerted opposite influences on the potential of progeny to produce Id-1. In addition, we observed that neonatal, but not adult, exposure to idiotypic and anti-idiotypic antibodies could induce enhanced Id-1. Both results suggested that idiotype specific cells within neonatal rats maintained a different accessibility or vulnerability to external manipulations than did cells from adult animals. This age dependent difference was responsible for the induction of different types of idiotype specific effector mechanisms.

Neonatal exposure to anti-Id-1 antibodies, Id-1 conjugated lymphocytes, antigen, or lymphocytes from Id-1 immune and Id-1 suppressed animals could each alter the individual's ability to produce Id-1. All of the above components can be present

within the maternal circulation and transferred to progeny via placenta and/or milk. It is likely, therefore, that the observed maternally induced influences resulted from a combination of these components.

Introduction

It is now clear that idiotype specific immune reactivity can regulate antigen induced immune responsiveness in vivo (for review see Rodkey 1980). The complexity of such a regulatory mechanism is apparent when one considers that B, T-helper (T_H) and T-suppressor (T_S) cells with either idiotypic or anti-idiotypic receptors (Janeway et al. 1975, Woodland and Cantor 1978, Hetzelberger and Eichmann 1978, Cosenza et al. 1979, Bottomly et al. 1979, Brown and Rodkey 1979, Wikler et al. 1979, Kim 1979, Bona and Paul 1979) have been identified and some found to co-exist in the same individual at the same time (Bottomly et al. 1979, Sy et al. 1980, Hirai and Nisonoff 1980). The regulation of idiotype production is further complicated because a single idiotypic network is not autonomous within an individual, but rather is influenced by cross-reactive and non-idiotype-specific regulatory mechanisms. Therefore, when studying idiotype production by an individual in response to immunization with a particular antigen, one is actually monitoring the final result of the interactions of many regulatory networks. It is evident why it has been difficult to assess the importance of idiotypic interactions in the regulation of immune responsiveness in vivo.

We previously reported that antigen-induced, idiotype specific immune reactivity of female rats can bias antigen induced production of the same cross-reactive idiotype by their progeny at maturity (Olson and Leslie 1981). These results emphasized the potential of the idiotypic network to permanently regulate immune responsiveness in vivo. It was also evident that maternal regulation of progeny idiotype production, as with the regulation of idiotype production, in general, is complex. This was particularly evident from the observation that females with phenotypically identical idiotype suppressed states appeared to have the potential to differentially alter progeny idiotype production, depending upon the age in which idiotype suppression was induced in the females.

It was the purpose of the current studies to further clarify the mechanisms responsible for the maternally induced influences on progeny idiotype synthesis. Two significant observations were made. Firstly, neonatal exposure to idiotypic influences, either via maternal transfer or exogeneous injections, can induce significant positive or negative effects on idiotype production, if the appropriate signal is delivered. This is in contrast to idiotypic manipulations of adult animals which do not favor the enhancement of idiotype production. Secondly, idiotypic and anti-idiotypic antibodies, idiotype bearing lymphocytes and antigen all have the potential to alter idiotype production in vivo if injected neonatally, suggesting that the observed maternally induced alterations in idiotype production of progeny most likely reflect multiple interacting influences.

The idiotype system used in these studies was a public idiotype (Id-1) associated with rat anti-Group A streptococcal carbohydrate (anti-SACHO) antibodies, which was previously described (Stankus and Leslie 1974). The results obtained using the Id-1 idiotype were related to idiotype regulatory mechanisms defined by other laboratories.

Materials and Methods

Animals. Inbred Fischer 344 (F344) rats, a low responding strain to SACHO, were purchased from Simonsen Laboratories, Gilroy, California. HPR rats, F_{12-18} generations, were inbred in our laboratory for high responsiveness to SACHO as described previously (Stankus and Leslie 1975, Olson and Leslie 1981).

Immunization with Group A streptococci. GASV was prepared from a lyophilized stock culture of Group A streptococcus, strain J17A4 (Stankus and Leslie 1975). To induce anti-SACHO antibodies, HPR or F344 rats were immunized at 3 months of age by either intravenous (iv) or intraperitoneal (ip) injections of 1 mg of GASV given 3 times per week on consecutive days for 2 to 3 weeks. Animals were bled 5 days after the last weekly injection.

Quantitation of anti-SACHO antibodies. Total anti-SACHO antibodies were quantified by radioimmunoassay (RIA) using a modified Farr technique as has been previously described (Olson and Leslie 1981). Levels of precipitating anti-SACHO were determined by "low-salt" reverse radial immunodiffusion (RRID) as described earlier (Leslie and Hattier 1974).

Quantitation of Id-1. Id-1 was quantified by a competitive inhibition RIA using heterologous anti-Id-1 prepared against the $F(ab')_2$ fragment of specifically purified anti-SACHO antibodies that were >90% Id-1 positive (Olson and Leslie 1981).

Neonatal injections.

a. Injection of Id-1 immune serum. To induce autologous anti-Id-1 serum, HPR rats were immunized over a 4 month period with 500 µg of Id-1 IgG. Initial injections were given in Freund's complete adjuvant, in the hind foot pads and subcutaneously. Later injections were given subcutaneously in incomplete adjuvant or saline. Induction of autologous anti-Id-1 was monitored by Ouchterlony analysis (Leslie and Hattier 1974). Immune sera collected from a single female (2-2) within 3 weeks of birth of her progeny, were pooled and aliquots injected ip, 4 times into neonatal HPR rats (born to normal females) during the first 9 days of life. A total of 300 µl of serum was injected into each neonate. Control animals received the same volume of normal HPR serum via the same injection protocol.

b. Injection of $Id\text{-}1^+$ anti-SACHO antibodies or Id-1 conjugated cells. Neonatal rats, born to normal females, were given one ip injection of the indicated Id-1 specific antibodies or Id-1 conjugated syngeneic thymocytes within 24 hours after birth. Id-1 was conjugated to the thymocytes using N-ethyl-N'-(3'-dimethylaminopropyl carbodiimide) (Sigma, St. Louis, MO) as described by Dohi and Nisonoff (1979). A total of 15.4 µg of specifically purified anti-SACHO antibodies (>90% $Id\text{-}1^+$ by RIA was coupled to 1.4×10^7 thymocytes.

c. Neonatal injection of antigen. Neonatal injections of GASV, SACHO or 0.01 M phosphate buffered saline (PBS) were given ip within 24 hours of birth. The indicated antigen was diluted in PBS, and a 50 µl volume was injected into each neonate.

Neonates were also fed 1 mg of GASV via intubation within 24 hours of birth.

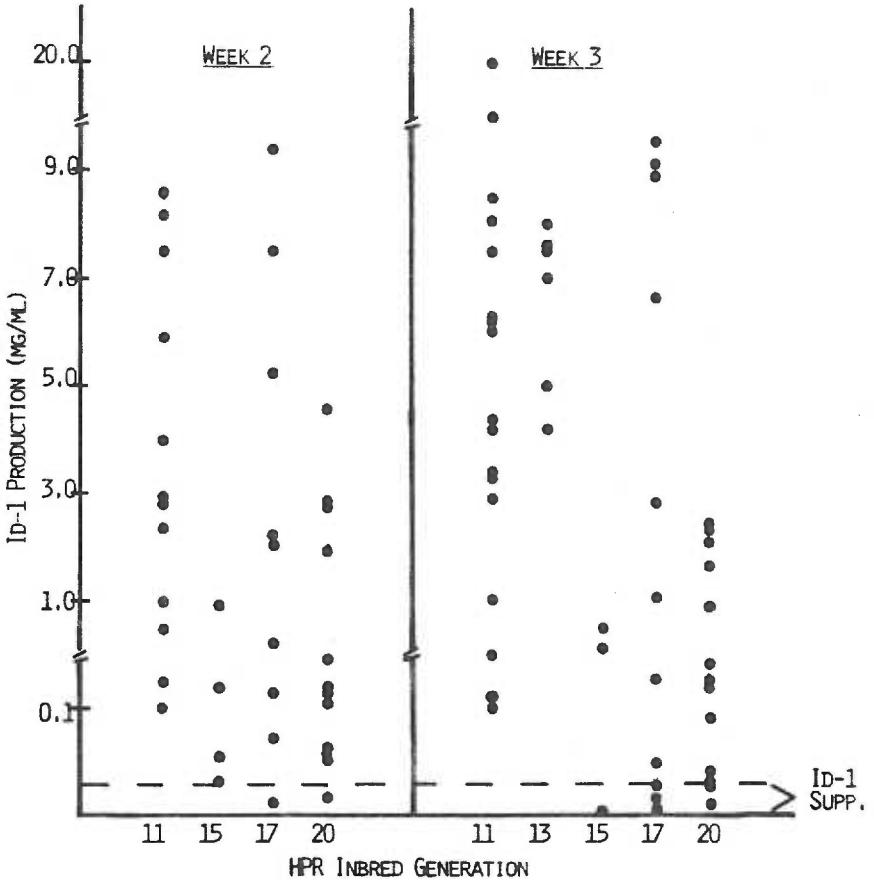
Spleen cell transfers. Spleens from Id-1 suppressed or Id-1 immune females were minced, cell suspensions made and washed in Minimum Essential Medium (MEM: Grand Island Biological Co., Grand Island, NY). The cells were resuspended for 5 minutes at room temperature in 9 parts 0.83% ammonium chloride:1 part 0.2 M Tris buffer (pH 7.4) to lyse red blood cells. Leukocytes were then washed 2 times in MEM, resuspended in MEM to a concentration of 1×10^7 per 50 µl and 50 µl injected ip into newborn rats.

Statistical analysis. Where indicated, results were analyzed statistically with a direction two-sample t-statistical evaluation test.

Results

Idiotype production by progeny of Id-1 suppressed female rats. In agreement with our previous observation in F344 rats (Olson and Leslie 1981), GASV immunized progeny of nonimmune HPR parents of the same inbred generation exhibit considerable heterogeneity in their ability to produce Id-1; this includes a small number of animals which are naturally suppressed for Id-1 synthesis (Figure 1). Maternal Id-1 specific immune reactivity, however, has been shown to restrict this heterogeneity (Olson and Leslie 1981).

Figure 1. Id-1 production of GASV immunized HPR rats of the indicated generation born to non-immune HPR parents. Animals were immunized with GASV at 3 months of age. Bleedings taken 2 and 3 weeks after initiation of the immunization protocol are indicated. Animals producing Id-1 concentrations below that represented by the dashed line throughout the 3 week immunization protocol are defined as Id-1 suppressed.


WEEK 2
WEEK 3
20.0
9.0
7.0
5.0
3.0
1.0
0.1
ID-1 PRODUCTION (MG/ML)
ID-1 SUPP.
11
15
17
20
11
13
15
17
20
HPR INBRED GENERATION

HPR female rats suppressed for Id-1 production by treatment as neonates or adults with anti-Id-1, which have equally suppressed levels of Id-1 and comparable concentrations of anti-SACHO antibodies (Table 1), were bred and their progeny were immunized with GASV at 3 months of age. Either non-immune males, or a single male, served as father for each female in a study, in order to eliminate possible paternal influences. Id-1 and anti-SACHO production by progeny were analyzed and compared to that of progeny of either non-immune or control HPR females born at approximately the same time. To avoid complications due to possible differences in vaccine preparations and interassay variations, all animals in a given study were immunized with the same GASV preparation and their sera were quantified together in the same assay.

As shown in Table 1, the potential for progeny of Id-1 suppressed females to produce Id-1 was significantly altered from that of control progeny of the same generation. More than 50% of the progeny (7 of 13) of females suppressed for Id-1 production as adults were Id-1 suppressed. This is in contrast with our previous results showing that progeny of females suppressed neonatally for Id-1 production had a significantly enhanced potential to produce Id-1 (Olson and Leslie 1980 and represented in Table 1). The ability of Id-1 specific regulatory factors from neonatally Id-1 suppressed females to induce enhanced Id-1 production in progeny was confirmed by foster mother studies (Olson and Leslie 1981). The opposite influences on progeny induced by females suppressed for Id-1 production at different ages, suggest that different mechanisms can be associated with maintenance of identical Id-1 suppressed states.

Table 1. Analysis of Id-1 and anti-SACHO production by progeny of HPR females neonatally or adult suppressed for Id-1

	Mothers:			Progeny:		
	Treatment	Id-1[a] (mg/ml)	Anti-SACHO	Id-1[b] (mg/ml)	Anti-SACHO[c]	Id-1:SACHO ratio
F_{17}	Id-1 suppressed as adults GASV immune (female 102 & female 1-4)	0.01	3.1	1.8 ± 1.1 (0.01;0.01;0.01;0.01; 0.02;0.02;0.03;0.09;0.9; 1.1;1.4;1.5;15.5)[d]	5.9 ± 1.2	0.53 ± 0.22
	Controls: non-suppressed GASV immune (female 3 & female 4)	4.4	5.4	1.7 ± 0.8 (0.01;0.05;0.1;0.2;0.4; 1.0;1.0;1.5;2.7;13.5)	3.5 ± 0.7	0.55 ± 0.19
F_{15}[e]	Id-1 suppressed neonatally GASV immune (female 3-2 & female 3-3)	0.01	5.4	1.7 ± 0.3[f] (0.02;0.1;0.3;0.3;0.5; 0.7;0.7;1.4;1.4;1.8;1.8; 2.4;2.4;5.1;5.1;6.7)	1.6 ± 0.3[f]	1.38 ± 0.17[f]

Table 1 - Continued

	Controls: non-suppressed non GASV immune (n=2)	n.d.[g]	n.d.	0.7 ± 0.2[f] (0.02;0.02;0.09;0.3;0.7; 1.1;1.5;1.9;3.6)	4.0 ± 0.7	0.51 ± 0.2[f]
F_{18}	Id-1 suppressed neonatally GASV immune (female 7-1)	0.01	4.4	3.6 ± 1.1 (0.02;0.2;1.4;2.1;2.4; 3.1;3.6;4.0;8.1;11.5)	2.9 ± 0.8	1.56 ± 0.42[f]
	Controls: non-suppressed GASV immune (female 6-5 & female 6-8)	3.4	4.6	1.7 ± 0.7 (0.01;0.01;0.02;0.02; 0.02;0.04;0.04;0.05;0.2; 0.3;0.3;0.3;0.4;0.5;1.0; 1.0;1.2;1.2;6.7;8.0;8.7; 10.0)	4.2 ± 0.8	0.46 ± 0.17[f]

[a]Id-1 quantified by RIA, precipitating anti-SACHO quantified by RRID.

[b]Id-1: $\bar{x} \pm$ SEM after 2 or 3 weeks of GASV immunization. Id-1 concentrations of the individual animals are listed in brackets.

Table 1 - Continued

[c]Anti-SACHO: $\bar{x} \pm$ SEM after 2 or 3 weeks of GASV immunization are indicated.

[d]Underlined numbers represent Id-1 suppressed animals. Id-1 suppression is defined as producing <30 µg/ml of Id-1 throughout the 3 week immunization protocol.

[e]F_{15} and F_{18} studies adapted from Leslie and Olson 1981.

[f]$p<0.05-0.001$

[g]n.d. = not determined.

To increase our understanding of these mechanisms we analyzed the potential of the humoral and the cellular arm of the immune system to alter Id-1 production.

The effect of maternal humoral immune reactivity on neonatal animals. We previously observed that the neonatal injection of heterologous anti-Id-1 antibodies could induce Id-1 suppression in 50% or more of the treated animals (Olson et al., in preparation). The production and transfer of autologous anti-Id-1 antibodies by females during gestation and the neonatal developmental period may have, via a similar mechanism, been responsible for inducing a suppressive influence on progeny idiotype production.

To determine if circulating autologous anti-Id-1 had the ability to alter Id-1 production of progeny, we extensively immunized HPR rats with $Id\text{-}1^+$ IgG. Progeny born to these females had significantly suppressed Id-1 production (63±23 μg/ml Id-1) as compared to progeny of normal rat IgG immune females (3325±1046 μg/ml Id-1), suggesting that an Id-1 specific influence had been induced. One such Id-1 immune female, 2-2, was bled several times within 3 weeks of parturition. Sera collected at these times were pooled and injected ip into neonatal HPR rats born to a nonimmune female. The serum used for these injections contained no detectable Id-1 by RIA nor anti-Id-1, as determined by a less sensitive technique, Ouchterlony gel-diffusion analysis. Littermates receiving the same volume of normal HPR serum served as controls. All animals were immunized with GASV iv at 3 months of age, and their Id-1 and anti-SACHO concentrations were monitored. As shown in Table 2,

the proportion of animals suppressed for Id-1 production after receiving serum from Id-1 immune female 2-2 was comparable to the proportion we previously observed to be suppressed (50-67%) following neonatal injections of specifically purified heterologous anti-Id-1 (Olson et al. in preparation). This indicated that autologous Id-1 specific humoral immunity had the ability, when given to neonates, to suppress Id-1 production.

The influence of neonatal exposure to Id-1 on idiotype production of progeny. Id-1 was previously detected in sera of progeny of Id-1 producing GASV immunized females, but not in progeny of GASV immunized females lacking detectable Id-1 (Olson and Leslie 1981). Maximum concentrations of Id-1 were detected in neonatal rats approximately 15 days after birth which is coincident with peak transfer of maternal immunoglobulins in rats (Halliday 1955, Brambell 1970, Leslie in preparation). It is evident, therefore, that maternally derived Id-1 can be transferred to progeny, but does this have the potential to induce alterations in the ability of progeny to produce Id-1? To determine if maternally derived idiotype could influence idiotype production, we injected neonatal rats ip with increasing concentrations of Id-1. Two of six such studies are represented in Table 3.

In the first study, animals were injected neonatally with Id-1 or normal rat IgG. Following immunization with GASV ip at 3 months of age, the animals which received Id-1 produced significantly higher concentrations of Id-1 than did controls. This difference was not significant when the rats were immunized with GASV iv. The second study

Table 2. Id-1 production by HPR rats injected neonatally with serum from an Id-1 immune HPR female

Neonatal injection	No. of animals suppressed[a]	Id-1[b] (μg/ml)	Anti-SACHO (μg/ml)
300 μl serum from Id-1 immune female[c]	4/7	660±280	8620±840
Littermates injected with 300 μl normal HPR serum[c]	0/7	1030±400	7740±790

[a] Id-1 suppressed, defined as producing <30 μg/ml of Id-1 over the 3 week immunization schedule.

[b] Id-1 was quantified by RIA; precipitating anti-SACHO by RRID. Mean ± SEM are indicated.

[c] Neonates were injected with serum 4 times from 0 to 9 days of age. A total of 300 μl of serum was injected into each rat.

Table 3. Id-1 production by rats injected neonatally with Id-1

Study #	Strain	Neonatal Injection[a]	Route	(n)	GASV Immun. Id-1[b]	Anti-SACHO
1	HPR	Id-1 (100 µg)	ip	(4)	405±113[c] µg/ml	1302±341
		N. rat IgG (100 µg)	ip	(3)	120±63[c]	1982±17
	HPR	Id-1 (100 µg)	iv	(4)	1160±746	1685±951
		N. rat IgG (100 µg)	iv	(5)	717±426	1814±1081
2	F344	Id-1$^+$ IgG (2 µg)	ip	(5)	365±250	nd
		(20 µg)	ip	(4)	1561±806	nd
		(100 µg)	ip	(3)	740±367	nd
		(200 µg)	ip	(4)	277±151	nd
		Id-1$^-$ IgG (2 µg)	ip	(5)	497±296	nd
		(20 µg)	ip	(5)	718±347	nd
		(100 µg)	ip	(9)	651±342	nd

Table 3. (Continued)

Study #	Strain	Neonatal Injection[a]	GASV Immun. Route	(n)	Id-1[b]	Anti-SACHO
3	F344	$Id\text{-}1^+$ IgG (20 μg) + anti-Id-1 (100 μg)	ip	(5)	1068±178	nd
		$Id\text{-}1^-$ IgG (20 μg) + N. rabbit IgG (100 μg)	ip	(10)	724±240	nd
4	F344	15.4 μg Id-1 conjugated to $1.4x10^7$ thymocytes	iv	(7)	5592±733[c]	2839±1544
		15.4 μg Id-1 + $1.4x10^7$ thymocytes	iv	(5)	3118±1179	2195±1153
		15.4 μg N. rat IgG conjugated to $1.4x10^7$ thymocytes	iv	(6)	1849±830[c]	2971±1113

[a]The indicated protein or cells were injected within 24 hours of birth.

[b]Refer to Table 1 for methodology and terminology

[c]$p < 0.05 - < 0.025$.

indicates that influences on idiotype production in the neonate had a tendency to vary depending upon the amount of Id-1 injected. In 4 additional studies, however (data not shown), neonatal injections of Id-1 at amounts ranging from 2-350 μg did not induce significant changes in the animals' ability to produce Id-1. These studies emphasized the overall ineffectiveness of ip injections of Id-1 alone in altering an individual's potential to synthesize Id-1.

In a third study (Table 3), neonatal injection of a mixture of Id-1 and anti-Id-1 could induce enhanced Id-1 production, but again this was not a consistent observation. What appeared to be the most effective means of inducing Id-1 enhancement, however, was to inject neonatal animals ip with Id-1-conjugated homologous thymocytes (Table 3, Study 4). This suggests that maternally derived Id-1 specific lymphocytes may play a more important role than humoral immune factors in the potentiation of idiotype production by progeny.

The influence of neonatal exposure to antigen on idiotype production. It is difficult to distinguish whether maternally induced alterations in progeny immune responsiveness in vivo result from the transfer of immunological regulatory factors or are a result of direct influences induced by passively transferred antigen. Both are likely present within the maternal circulation and each has the potential to be transferred to the neonate (Solomon 1972, Kindred and Roelants 1974, Auerbach and Clark 1975, Stern 1976, Sasake 1977, Gill et al. 1977). We previously noted (Olson and Leslie 1981) that there was no correlation between the time of last injection of antigen into females and subsequent

Id-1 production by the animals. When the effect of neonatal exposure to antigen on subsequent immune responsiveness was examined directly, we observed that some of the animals receiving GASV neonatally (either ip or intragastrically) had a tendency for increased Id-1 production. This increase, however, appeared to coincide with an overall stimulation of total anti-SACHO antibody production and the increase in Id-1 was not statistically significant. The results from such studies in HPR and F344 rats are shown in Table 4.

Transfer of cells from Id-1 suppressed or Id-1 immune females. To examine the role of immune cells in the induction of Id-1 specific regulatory influences, HPR neonatal rats were injected with the spleen cells of Id-1 immune or Id-1 suppressed HPR rats. In all studies 1×10^7 unfractionated, ammonium chloride treated spleen cells were injected ip into neonatal rats within 24 hours of birth. The rats in these studies were born to normal HPR females, and littermates, injected with MEM, served as controls. Animals were immunized with GASV iv at 3 months of age.

As shown in Table 5, the transfer of unfractionated spleen cells from Id-1 suppressed females increased the frequency of Id-1 suppression in the cell recipients above that of non-injected littermates (Study 1). An increase in the frequency of suppression, however, was not observed when spleen cells from non-suppressed, GASV immune females were transferred to neonatal animals (Study 2). The fact that neither Id-1 suppression nor Id-1 enhancement was observed in this study favors the possibility that idiotype specific influences, rather than

Table 4. The influence of neonatal treatment with antigen on Id-1 and anti-SACHO production

Strain	Neonatal treatment	Concentration of:[a] Id-1 (μg/ml)	Concentration of:[a] Anti-SACHO (μg/ml)
HPR	158 μg GASV (ip)[b] n=6	3656±2369	3577±813
	40 μg SACHO (ip)[b] n=5	2615±1988	3990±3170
	PBS (ip) n=5	1683±1273	2890±913
F344	1 mg GASV (fed) n=6	3084±1606	3112±1030
	Non-treated littermates n=4	1194±949	1667±583
F344	50 μg GASV (ip) n=6	2841±1232	2667±773[c]
	Non-treated littermates n=4	506±461	536±133[c]

[a]All animals were immunized with GASV iv at 3 months of age. Weeks 2 or 3 after the initiation of GASV immunization are indicated. Id-1 and anti-SACHO were quantified by RIA and RRID, respectively.

Table 4 - Continued (Footnotes)

[b]40 µg of SACHO is equivalent to the amount of carbohydrate in 158 µg of GASV.

[c]$p<0.05$

Table 5. Id-1 production by HPR rats receiving cells neonatally from Id-1 suppressed or Id-1 immune HPR donors

Study No.	Cell source[a]	Concentration of:[b] Id-1 (mg/ml)	Anti-SACHO
1	Cells from Id-1 suppressed HPR females (n=2)[d]	0.02;[c]0.02;0.02;0.02; 0.03;0.09;0.2;5.9;7.3	4.1 ± 1.0
	MEM injected littermates	0.04;0.06;0.08;0.2; 0.3;0.4;0.5;2.4;9.0	1.8 ± 0.6
2	Cells from non-Id-1 suppressed HPR females (n=2)[d]	0.8;2.2;3.9;5.0;5.4	1.9 ± 0.7
	MEM injected littermates	0.1;0.5;2.0;2.1;12.0	1.4 ± 0.6
3	Cells from Id-1 immune 1-3[d]	0.2;11.0;36.0;38.0[e]	10.0 ± 2.8
	MEM injected littermates	0.1;0.6;0.8;7.2[e]	9.8 ± 2.8
4	Cells from Id-1 immune 2-2[d]	0.02;0.08;0.1;0.4;6.8	4.7 ± 2.3
	MEM injected littermates	0.3;0.8;5.9;6.1	5.0 ± 2.1

Table 5 - Continued

Footnotes

[a] 1×10^7 unfractionated spleen cells were transferred to normal HPR neonates within 24 hours of birth.

[b] Id-1 quantified by RIA, anti-SACHO RRID. Bleedings taken 2-3 weeks after the initiation of the GASV immunization protocol are represented. Id-1 concentrations of individual animals are represented and $\bar{x} \pm$ SEM of anti-SACHO concentrations are shown.

[c] Underlined concentrations indicate Id-1 suppressed animals. Id-1 suppressed defined as producing <0.03 mg/ml of Id-1 during the 2-3 weeks GASV hyperimmunization protocol.

[d] Donor antibody concentrations at the time of cell transfer:

Study 1: Id-1 $\bar{x}$ = 0.003 mg/ml; Anti-SACHO $\bar{x}$ = 10 mg/ml.

Study 2: Id-1 $\bar{x}$ = 0.5 mg/ml; Anti-SACHO $\bar{x}$ = 6.1 mg/ml.

Study 3 & 4: Id-1 $\bar{x}$ = 0 mg/ml; Anti-SACHO $\bar{x}$ = 0 mg/ml.

[e] $p<0.05$.

allogeneic influences, were responsible for the increased incidence of idiotype suppression in the recipients of cells from Id-1 suppressed females. It is interesting to note that in study 1, anti-SACHO production was not concomitantly suppressed. In contrast, anti-SACHO concentrations appeared to be enhanced in the Id-1 suppressed recipients.

We also injected neonatal HPR rats with cells from two HPR rats extensively immunized with Id-1, to induce autologous Id-1-specific immunity. As shown in Table 5 (Study 3), cell recipients had significantly enhanced Id-1 production. However, in study 4, spleen cells from an animal receiving the identical Id-1 immunization protocol as that of the donor of study 3, and indistinguishable in Id-1 and anti-Id-1 production, were unable to transfer a significant influence to neonates.

It is apparent from these studies that the transfer of cells from Id-1 suppressed or Id-1 immune animals had the potential to suppress or enhance Id-1 production of neonatal recipients. Further studies are required to clarify the cell populations responsible for these influences and to relate this to the cell populations present during the perinatal period and associated with maternally induced alterations in progeny idiotype production.

Discussion

The measurable in vivo immune response represents the final result of the activation of a network of interacting cells. For the production of a specific idiotype, these cell populations include B, T_H, and

T_S cells, each having idiotype or anti-idiotype cell surface receptors (Janeway et al. 1975, Woodland and Cantor 1978, Hetzelberger and Eichmann 1978, Bottomly et al. 1978, Cosenza 1979, Brown and Rodkey 1979, Wikler et al. 1979, Kim 1979, Bona and Paul 1979, Bottomly and Mosier 1979, Fung and Kohler 1980a). With the increasing evidence that different idiotype specific T-regulatory cells can co-exist in the same individual (Bottomly et al. 1978, Sy et al. 1980, Hirai and Nisonoff 1980), it becomes clear that we must understand the interaction and proportionate contribution of these different cell populations in regulating idiotype production in situ if we are going to be able to selectively manipulate immunologic responsiveness. The present studies were designed to increase our understanding of the mechanisms which regulate the production of idiotype in vivo. We wished to compare maternal influences on progeny idiotype production to idiotype specific manipulations of neonatal animals. Previously, we proved that the maternal immune system had a significant effect on the production of idiotype by progeny (Olson and Leslie 1981).

Our studies reinforce the complex nature of idiotype specific regulatory mechanisms; e.g. Id-1 suppressed females, phenotypically identical with respect to their Id-1 and anti-SACHO production, induced opposite influences on progeny idiotype production, depending upon the age at which they were Id-1 suppressed. Differences between neonatal and adult mechanisms of induction of Id-1 suppression were also apparent by the consistently higher percentage of animals becoming Id-1 suppressed following injections of anti-Id-1 as adults (83-86%), as compared with those

suppressed neonatally (50-67%) (Olson et al. in preparation). These results suggest that different regulatory mechanisms are associated with the induction and maintenance of Id-1 suppression in neonatal and adult rats.

Age dependent differences in idiotype specific regulatory mehanisms have been reported by other investigators. Strayer et al. (1975) and Augustin and Cosenza (1976) observed that anti-TEPC-15 idiotypic antibodies induced chronic idiotype suppression (lasting at least 26 weeks) when injected ip into neonatal BALB/c mice. Only acute suppression (< 3 weeks) was induced when anti-idiotypic antibodies were injected into adult mice. Similar results were observed by Bona et al. (1979) for the suppression of IdX associated with mouse anti-inulin antibodies. Recently, Fung and Kohler (1980a, b) reported that neonatally induced suppression of TEPC-15 idiotype was associated with arrested development of idiotype positive B cells. This lack of B cell development was related to the presence of T_S cells effective only against immature cells, and possibly an insufficiency of idiotype specific T_H cells. In contrast to the mechanism of neonatally induced suppression, idiotype suppression in adults appeared to result from a transient blockage of antigen receptors on lymphocytes.

To increase our understanding of Id-1 production, we examined, separately, the role of humoral and cellular factors in the regulation of its synthesis. Neonatal injections of anti-Id-1 specific serum from Id-1 immune female 2-2 (a mother previously giving birth to progeny significantly suppressed for Id-1 synthesis), transferred Id-1 suppression to >50% of the recipients. This was comparable to the percentage

of animals suppressed for Id-1 following the neonatal injection of heterologous anti-Id-1 antibodies (Olson et al. in preparation), and supports the ability of maternally derived humoral immunity to suppress Id-1 production.

Our previous studies (Olson and Leslie 1981) showed that maternal Id-1 is transferred to progeny. The question then is, does neonatal exposure to Id-1 alter the potential to produce Id-1? To answer this question, Id-1 or Id-1 in association with anti-Id-1 was injected into neonatal rats, and the animal's ability to produce Id-1 as adults was assessed. No consistent alterations in the animal's ability to produce Id-1 were apparent, regardless of the concentration of Id-1 injected. A predictable enhancement of Id-1 production, however, was attained by injecting neonatal rats with Id-1 conjugated thymocytes.

Previous studies have shown that idiotype production can be stimulated by prior exposure to idiotype (Janeway et al. 1975, Woodland and Cantor 1978, Bottomly et al. 1980). In addition, anti-idiotypic antibodies have also been shown to induce enhanced idiotype production (Black et al. 1976, Julius et al. 1977, Owen et al. 1977, Hetzelberger and Eichmann 1978). In order to explain such results, one would have to postulate either the existence of two idiotype specific regulatory cells having complementary receptors, or the existence of one cell which requires idiotype-anti-idiotype interactions to be stimulated. In this regard a T_H cell with an idiotype-specific receptor has been identified by Bottomly et al. and this T cell requires interaction with idiotype or idiotype bearing B cells for activation.

To determine the ability of cells, as opposed to humoral immune factors, to alter Id-1 production, neonatal rats were injected ip with unfractionated spleen cells form Id-1 immune or Id-1 suppressed syngeneic rats. More than half of the recipients of cells from Id-1 suppressed rats were Id-1 suppressed, whereas one litter receiving cells from and Id-1 immune female had significantly enhanced Id-1 production. These results indicate that neonatal exposure to immunoregulatory cells has the potential to either enhance or suppress idiotype production. The exact influence induced may depend upon the functional characteristics of the cells transferred (i.e. B, T or a subpopulation of T cells) as reported by Eig et al. (1977) and Sy et al. (1981), or on the recipients interpretation of the signal delivered by the transferred cells, as observed by Rohrer et al. (1981).

Maternally derived cellular and humoral immune factors can be transferred during gestation and/or the neonatal developmental period. Since both have the potential to induce alterations in progeny idiotype production, the phenotypic influences of maternal idiotype specific immune reactivity on progeny immune responsiveness most likely reflect influences induced via both mechanisms. We, in agreement with others (Kindred and Roelants 1976, Halsey and Benjamin 1976, Gill et al. 1977) have shown that maternally derived antigen can also alter immune responsiveness in vivo. Our results, however, indicate that idiotypic influences can dominate antigen induced influences on Id-1 production.

Unlike the induction of idiotype suppression there is a noticeable paucity in the reports of induction of enhanced idiotype production in vivo. As mentioned above, neonatal exposure to Id-1 conjugated

lymphocytes, anti-Id-1 antibodies or maternally derived Id-1 specific components can enhance the potential of the individual to produce Id-1. In contrast, we have never been able to enhance Id-1 production in adult animals, although comparable methods were employed. In addition, the fact that the neonatal injection of Id-1 coupled to cells serves as a more efficient means of inducing Id-1 production than Id-1 alone, suggests that signals required for the enhancement of Id-1 production are more complex than those required for triggering suppression, as previously suggested by Woodland and Cantor (1978). For this reason, even though enhancement and suppression of Id-1 production can be induced by neonatal manipulations, functional activation of suppression is more readily induced than enhancement. In fact, evidence that cells associated with enhanced Id-1 production have been activated, but remain quiescent in a suppressed animal, may explain why neonatally Id-1 suppressed females transfer idiotype enhancing immunity to their progeny.

It appears that during maturation, suppressor cells remain vulnerable to idiotypic manipulations, while helper cells, although functionally active are less vulnerable to extrinsic manipulations and prefer to maintain a pre-established regulatory pattern. (A summary of these age dependent differences as related to the regulation of Id-1 production is shown in Table 6.) The apparent autonomy of the helper population in mature animals may reflect a sequestration of these cells to peripheral sites or the acquisition of a refractory state. Consequently, attempts to exogenously regulate idiotype production in adult animals will either lead to idiotype suppression, or be unsuccessful. Unlike

Table 6. Summary of differences between regulatory influences on Id-1 production in neonatal and adult rats

Treatment	Effect on Id-1 production	
	Neonatal Injection	Adult injection
anti-Id-1 (ip) heterologous	50-67% suppressed[a]	83-86% suppressed
or homologous	20-33% enhanced[b]	0% enhanced
controls:	0-20% suppressed	17% suppressed
Id-1 or Id-1 and	2 studies: enhanced[c]	no effect
anti-Id-1 (ip)	4 studies: no effect	
Id-1 conjugated to	enhanced[c]	no effect
thymocytes		
	Cells from neonatally suppressed	Cells from adult suppressed
Spleen cells from suppressed	60% suppressed	50% suppressed
females transferred to	20% enhanced	0% enhanced
neonates		
controls:	0% suppressed	0% suppressed

[a]Suppressed: defined as producing <30 µg/ml of Id-1 for 2-3 week immunization protocol.

[b]Enhancement: defined as producing higher Id-1 concentrations than any of the control animals.

[c]Significantly enhanced at a p value of <0.05.

maternally or neonatally induced influences on idiotype production, one would predict that it would be very difficult to induce enhanced idiotype synthesis in adult animals.

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PAPER 3

The Assessment of Anti-Idiotype Antibodies as Effective Immunoregulatory Probes in vivo

Abstract

In order to increase our understanding of the potential to use anti-idiotypic antibodies as immune modulators in vivo, we extensively analyzed influences induced by one such antibody (anti-Id-1) following its administration to animals of different ages, genetic backgrounds, and immunological histories.

Id-1 is an inter-strain idiotype associated with rat anti-Group A streptococcal carbohydrate antibodies. The intraperitoneal (ip) injection of anti-Id-1 antibodies, prepared against Id-1^+ antibodies from an HPR rat could effectively induce long-term idiotype suppression in all tested strains of rats, regardless of the age at the time of treatment, the Rt-1 haplotype or IgG_{2c} or κ-chain allotype. Total anti-streptococcal antibodies were not suppressed by this treatment.

Although long-term suppression could be induced at any age, the percentage of animals suppressed following neonatal injections was consistently less than that following adult injections of anti-idiotypic antibodies. In addition, neonatal injections of anti-Id-1 or Id-1 with anti-Id-1 appeared to enhance Id-1 production in a minority of the animals. Similar treatment of adult animals never increased Id-1 synthesis, suggesting that cells associated with enhanced Id-1 production in older animals are either refractory to activation-differentiation signals and/or are sequestered and no longer accessible by iv or ip routes of administration of the probe. Auto-anti-Id-1 immunity induced by immunizing adult rats with heavy, light, $F(ab')_2$ fragments or whole IgG molecules could also induce an Id-1 suppressed state.

We were not able to induce significant Id-1 suppression if animals were immunized with antigen prior to the injection of anti-Id-1. There

was evidence, however, that such treatment might lead in time to the development of an idiotype specific immunity.

Introduction

The primary advantage of idiotypic manipulations over other exogenous immunological manipulations in altering immune responsiveness is their precision. The administration of homologous or heterologous anti-idiotypes or idiotype conjugated lymphocytes can selectively suppress or enhance idiotype production without affecting overall immune responsiveness (Eichmann and Rajewsky 1975, Nisonoff et al. 1977, Dohi and Nisonoff 1979, Sy et al. 1980). Similarly, idiotype and anti-idiotype bearing lymphocytes have been shown to be successful immune regulators in vivo (Janeway et al. 1975, Bottomly et al. 1978, Yamamoto et al. 1979, Dohi and Nisonoff 1979).

There are disadvantages associated with the use of idiotypic or anti-idiotypic antibodies as immunoregulatory probes in vivo. One disadvantage is believed to be their inability to effectively regulate immune reactivity against a heterogeneous immune response. This problem can be somewhat alleviated by the fact that anti-idiotypic antibodies have been successfully prepared against antibodies of a heterogeneous response (Brown et al. 1979, Fritz and Desjandins 1981, Zanetti and Bigazzi 1981), and that regulatory influences induced by anti-idiotype antibodies can be extended due to cross-reactivities amongst idiotypes(Eichmann and Kindt 1971, Kunkel et al. 1973, Stankus and Leslie 1974a, Forre et al. 1979). An additional concern of the use of

idiotypic probes in vivo is the need to have a complete understanding of the range of effects which will be induced by their in vivo administration. It is highly unlikely that the immune status of different individuals will be identical at the time of injection of a particular idiotypic probe since the immune system remains in a dynamic state. These fluctuations in immune responsiveness may redirect a specific anti-idiotypic reagent to functionally different cell populations, and one must be aware of the outcome of such redirections.

Regardless of these possible disadvantages, idiotypes provide clinicians a means with which to specifically alter immune responsiveness in a manner most effective for treatment of disease. In animal models, passive administration of anti-idiotypic antibodies or the induction of idiotype specific immunity in vivo has been shown to: a) suppress the growth of murine B cell lymphomas (Lanier et al. 1980), murine myelomas or plasmacytomas (Beaty et al. 1976, Abbas and Klaus 1977, Bridges 1978, Sakato et al. 1979, Lynch et al. 1981), and guinea pig leukemias (Stevenson 1977); b) inhibit or lessen the severity of autoimmune diseases such as tubulointerstitial nephritis in guinea pigs (Brown 1979) and autoimmune thyroiditis in rats (Zanetti 1981); and c) specifically suppress transplantation immunity of rats allowing prolonged graft survival (Binz and Wigzell 1976).

It was the intent of these studies to analyze extensively the potential of a specific anti-idiotypic antibody to regulate idiotype production in vivo, taking into consideration the age, genetic

background, and immunological history of the animal. For these studies we used a heterologous anti-idiotypic reagent prepared against a public idiotype (Id-1) associated with rat anti-Group A streptococcal carbohydrate (anti-SACHO) antibodies (Stankus and Leslie 1974a). All strains of rats tested have the potential to produce Id-1 following immunization with Group A streptococcal vaccine GASV (Stankus and Leslie 1976a). Depending upon the individual animal, heterogeneous to highly restricted anti-SACHO antibody populations can be induced by GASV (Stankus and Leslie 1974b), making this a versatile antigen for analyzing the feasibility of exogenous idiotypic regulation. Results gained from these studies emphasize both the advantages and limitations of using idiotypic-specific manipulations to regulate immune responsiveness in vivo.

Materials and Methods

Animals. Inbred Fischer 344 (F344) (Rt-1l, κ1b, γ2c1b), a low responding strain to SACHO, were purchased from Simonsen Laboratories, Gilroy, California. HPR rats (Rt-1b, κ1b, γ2c1b), originally derived from a Sprague-Dawley colony maintained at Tulane University Medical School, have been selectively brother-sister bred in our laboratory for more than eight years based upon the production of high levels of precipitating anti-SACHO antibodies (Stankus and Leslie 1975). August (Rt-1c, κ1b, γ2c1b) and Wistar Furth (Rt-1u, κ1a, γ2c1a) rats were obtained through the courtesy of Clarence Reeder at the Veterinary Resources Branch of the NIA, Bethesda, Md., and were further bred within our laboratory.

Immunization with Group A streptococci. A lyophilized stock culture of Group A streptococcus, strain J17A4, was used to prepare GASV, as previously described (Stankus and Leslie 1975). Primary immunizations with GASV were initiated at 3 months of age and consisted of 3 weeky injections of 1 mg, given on consecutive days for 2 or 3 weeks (standard GASV immunization protocol). All animals received GASV intravenously (iv) with the exception of F344 rats, which were immunized with GASV via the same protocol intraperitoneally (ip). The anti-SACHO antibodies induced are primarily of the γ2c subclass and bear kappa chains.

Preparation of anti-Id-1 antibody preparations. Heterologous anti-Id-1 antibodies were prepared against the $F(ab')_2$ fragment of Id-1 positive, anti-SACHO antibodies as previously described (Olson and Leslie 1981). Specifically purified (s.p.) anti-Id-1 antibodies were prepared by passage of monospecific anti-Id-1 over an immunoadsorbent column to which Id-1 positive, specifically purified anti-SACHO antibody molecules of restricted heterogeneity had been coupled. Antibodies were eluted from the column sequentially with 15 mM hapten (N-acetylglucosamine), 1, 2, or 3 M sodium thiocyanate (Stankus and Leslie 1976b). The 2 M thiocyanate eluates were used in these studies. The antigen binding capacity of anti-Id-1 preparations was calculated based upon its efficiency to bind a known quantity of an Id-1 specific radiolabeled antibody preparation.

Quantitation of Id-1. Id-1 was quantified by competitive inhibition radioimmunoassay (RIA) using a heterologous anti-Id-1 antiserum.

The RIA, which is described in detail in Olson and Leslie (1981), can detect 20 ng of Id-1 per sample.

Quantitation of anti-SACHO antibodies. Total anti-SACHO antibodies were assayed by RIA using a modified Farr technique (Olson and Leslie 1981). Levels of precipitating anti-SACHO were determined in "low" salt buffer by reverse radial immunodiffusion (RRID) as previously described (Leslie and Hattier 1974).

Neonatal injections of anti-Id-1. To suppress Id-1 production, neonatal animals were injected ip on days 1 and 3 with the indicated anti-Id-1 preparation. Occasionally additional injections were given as indicated in the legend of Table 1. The Id-1 preparation injected in conjunction with anti-Id-1 was a highly restricted Id-1 positive, specifically purified anti-SACHO antibody preparation obtained from a single HPR rat. Control animals were injected neonatally with normal rabbit IgG or normal rabbit serum, with or without normal rat IgG as indicated.

Injection of anti-Id-1 into adult animals. Normal HPR rats were injected at 3 months of age with the indicated anti-Id-1 preparation. The exact immunization protocol varied with the different studies as is detailed in the legend to Table 2. Control animals received normal rabbit IgG, normal rabbit serum or nothing as indicated.

In two other studies, adult HPR rats received their initial injection of anti-Id-1 2.5 or 10 months after the primary series of GASV immunizations. The quantity and details of administration of anti-Id-1 are described in Results. Following the injections of anti-Id-1 on days 1, 3 and 5, iv injections of 1 mg of GASV were given on days 7 and

12, with an additional 0.2 mg of GASV given on day 35 in one of the studies.

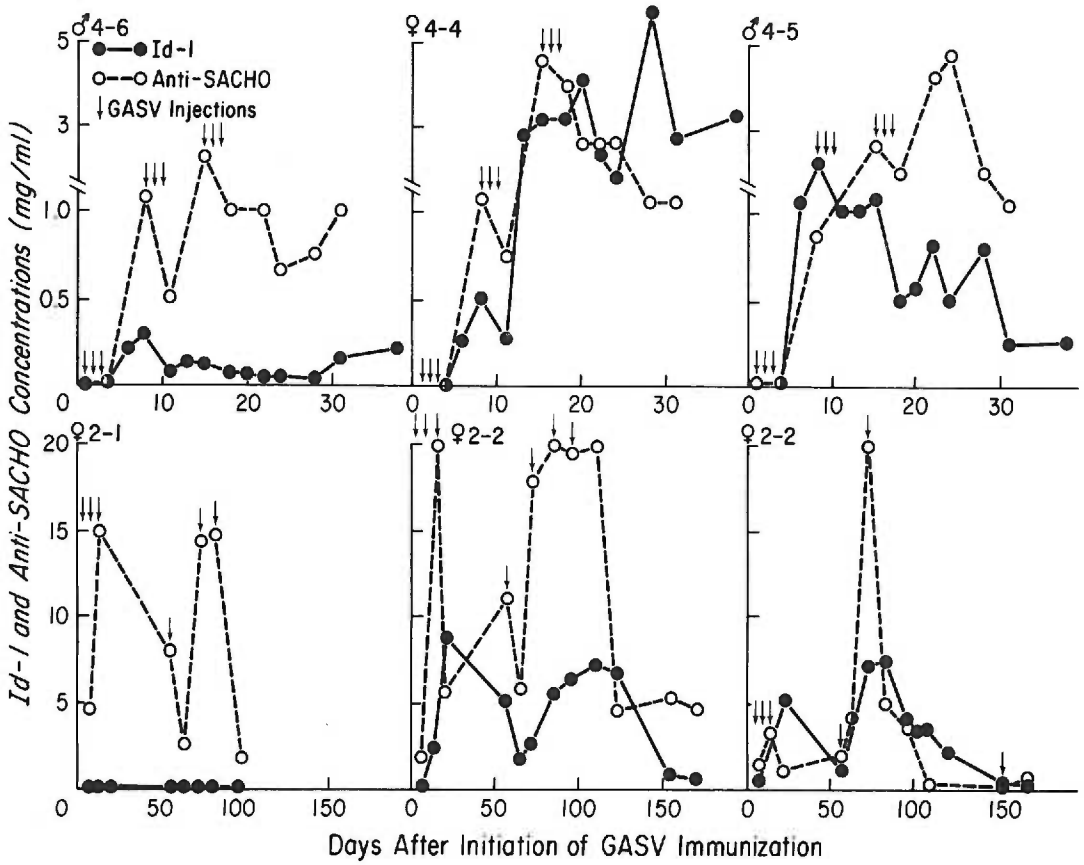
Induction of autologous anti-Id-1 immunity. To induce autologous anti-Id-1 immunity, F344 rats were immunized with either Id-1 positive IgG, $F(ab')_2$ fragments, heavy (H) or light (L) chains prepared as previously described (Stankus and Leslie 1974a). Rats were injected initially with 100 µg of the Id-1 preparation in FCA, distributed in the hind foot pads, and subcutaneously in multiple sites on the back. Additional subcutaneous injections of lower concentrations were given in FIA or saline during a 9 to 11 month period. A total of 480 µg of protein was injected into each animal. At this time the standard GASV immunization protocol was initiated.

Statistical analysis. Where indicated, results were statistically analyzed with a directional two-sample t-statistical evaluation test.

Results

Autologous regulation of Id-1 production. To increase our understanding of autologous mechanisms which may be regulating Id-1 production in vivo we monitored Id-1 concentrations in sera from GASV immunized HPR rats obtained: a) every other day during the 3 week GASV immunization protocol; and b) over a 6 month period following the initiation of the GASV immunization protocol. During those 6 months GASV was periodically injected as indicated in Figure 1. In both instances, the animals were from normal females and offspring were immunized with GASV beginning at 3 months of age. Id-1 production by 3 representative animals from each study is shown in Figure 1.

Figure 1. Id-1 and anti-SACHO production by GASV immunized HPR rats either: a) frequently bled during the GASV immunization protocol (upper graphs) or b) monitored over a 4-8 month period after the GASV immunization protocol was initiated. All animals are born to non-GASV immune females, with male 4-6, female 4-4 and male 4-5 being siblings as are female 2-1 and female 2-2. In the upper graphs precipitating anti-SACHO was quantified by RRID while in the lower graphs total anti-SACHO antibodies were quantified by RIA.


♂4-6
●—● Id-1
○--○ Anti-SACHO
↓GASV Injections
♀4-4
♂4-5
♀2-1
♀2-2
♀2-2
Id-1 and Anti-SACHO Concentrations (mg/ml)
Days After Initiation of GASV Immunization

The studies, firstly, emphasize the individual variation in Id-1 production. Secondly, although Id-1 and anti-SACHO production at times appeared to be synchronously regulated, it was evident in other individuals that both were regulated independently of each other. Thirdly, the relationship between Id-1 production and antigenic challenge was complex. Increases or decreases (e.g. male 4-5) in Id-1 production could occur following antigenic challenge and increases in Id-1 (e.g. female 4-4) could occur without antigenic challenge. These patterns of Id-1 production implicate the involvement of autologous Id-1 specific regulatory mechanisms in the regulation of Id-1 production in vivo.

Analysis of neonatally induced Id-1 suppression. Several laboratories have shown that idiotype production can be regulated by the administration of anti-idiotypic antibodies in vivo (Eichmann 1974, Nisonoff et al. 1977, Cosenza et al. 1977). Assuming the existence of Id-1 specific regulatory mechanisms, we examined the potential of anti-Id-1 antibodies prepared against the $F(ab')_2$ fragment of highly restricted $Id\text{-}1^+$ anti-SACHO antibodies from a single HPR rat to suppress Id-1 production when injected neonatally into the same or different strains of rats. Neonatal injections were given ip and initiated within 24 hours of birth. All animals were later challenged with GASV at 3 months of age. As shown in Table 1, a majority of the animals receiving anti-Id-1 antiserum or specifically purified anti-Id-1 neonatally were suppressed for Id-1 production. Anti-Id-1 together with Id-1 also served as an efficient means of inducing idiotype suppression; however, this influence was highly dependent on the concentrations of Id-1 and anti-Id-1

Table 1. Id-1 production by rats neonatally injected with anti-Id-1 antibodies

Strain	Neonatal Injection[a]	Concentrations: (μg/ml)[b] Id-1	Anti-SACHO	No. of animals[c] Id-1 suppressed
F344	100 μl anti-Id-1 serum	0;0;3;3; 5;5;9	n.d.[e]	7/7
	100 μg s.p. anti-Id-1[d]	0;9;36 >3500	n.d.	3/4
	100 μl anti-Id-1 serum + 2 μg Id-1	0;0;0	n.d.	3/3
	100 μg s.p. anti-Id-1 + 20 μg Id-1	620;920;945; 1167;1690	n.d.	0/5
	Controls[f]	5;8;12;82;88 186;310;422; 620;850;1600; 1700;1995	n.d.	3/13
HPR	100 μl anti-Id-1 serum	96;100	3630±320	0/2
	100 μg s.p. anti-Id-1	0;<5;1000; 3600	n.d.	2/4
	200 μg s.p. anti-Id-1	10;10;6100	5833±607	2/3
	200 μg s.p. anti-Id-1 + 20 μg Id-1	0;0;0;63 4610	4290±607	3/5
	100 μl anti-Id-1 + 350 μg Id-1	106;11300; 14200	9895±3455	0/3
	Control[f]	0;94;120; 168;240;445 500;1030;1450; 1450;2950;6000	4793±848	1/11

Table 1. Footnotes

[a]50 µl or 50 µg of anti-Id-1 was injected ip within 24 hours of birth. The injections were repeated on day 3. Two additional injections of anti-Id-1, every third day were given to animals receiving 200 µg of protein.

[b]Id-1 and anti-SACHO were quantified by RIA.

[c]Animals were defined as Id-1 suppressed if they produced <30 µg/ml of Id-1 throughout the 3 week immunization protocol.

[d]s.p.: specifically purified by passage over an Id-1^+ immunoabsorbent column.

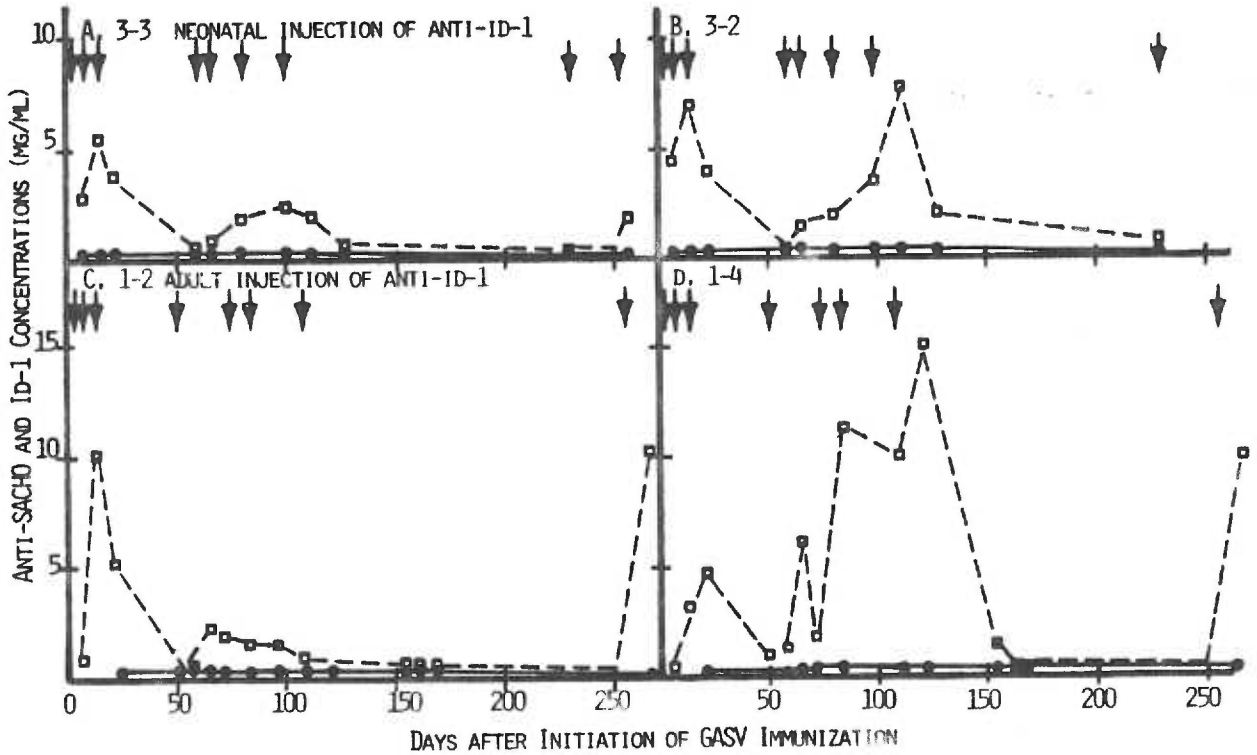
[e]n.d.: not determined.

[f]Control animals were injected with the appropriate rabbit and or rat serum proteins. No significant differences were noted in control groups of each strain and, for simplicity, were pooled in this table.

injected. Two examples of such studies for each strain are shown in Table 1. In F344 rats, 2 µg of Id-1 with 100 µl of anti-Id-1 serum suppressed Id-1 production, while 20 µg of Id-1 with 100 µg of anti-Id-1 enhanced Id-1 production. Similarly in the HPR studies 20 µg of Id-1 and 200 µg of anti-Id-1 suppressed Id-1 production in the majority of the animals, while 350 µg of Id-1 with 100 µl of anti-Id-1 serum led to enhanced Id-1 production in 2 of 3 animals. Thus in both strains of rats the higher the Id-1 to anti-Id-1 ratio, the greater the likelihood of increased Id-1 production. It should be noted, however, that even at high Id-1 to anti-Id-1 ratios it was difficult to consistently induce enhanced Id-1 production. Similarly, when Id-1 alone was injected into neonatal rats enhanced Id-1 production could be induced, but again enhancement was not consistently significant (Olson and Leslie, in preparation).

It is also apparent from Table 1 that even at the highest tested concentrations of specifically purified anti-Id-1 antibodies or Id-1 with anti-Id-1 antibodies a few animals resisted suppression. Interestingly some of these non-suppressed rats appeared to have enhanced Id-1 production. When suppression was induced, however, it was permanent. As exemplified in Figure 2, animals maintained an Id-1 suppressed state following intermittent rechallenges with antigen for a 1 year period, at which time the study was terminated. Neonatal exposure to anti-Id-1 had the potential to suppress Id-1 production in HPR, F344 and Wistar-Furth rats (data not shown for the latter strain). This emphasizes the potential of anti-idiotypic reagents to regulate idiotype

Figure 2. Duration of Id-1 suppression in neonatally or adult anti-Id-1 treated HPR rats. Neonatally suppressed rats (upper graphs) were injected with 200 μg of s.p. anti-Id-1, as outlined in Table 1. Adult anti-Id-1 treated rats (lower graphs) were injected ip at 3 months of age with 400 μg of s.p. anti-Id-1 as indicated in Table 2. Two representative animals are included for each study. The first 3 arrows represent the initial 3 week hyperimmunization protocol; the remaining arrows indicated the iv injection of 1 mg of GASV.


A. 3-3 NEONATAL INJECTION OF ANTI-ID-1
B. 3-2
C. 1-2 ADULT INJECTION OF ANTI-ID-1
D. 1-4
ANTI-SACHO AND ID-1 CONCENTRATIONS (MG/ML)
10
5
15
10
5
0
50
100
150
200
250
50
100
150
200
250
DAYS AFTER INITIATION OF GASV IMMUNIZATION

production of individuals with different histocompatibility antigens, and different immunoglobulin allotypes. In addition, although Id-1 production is suppressed, the animals can still elicit an immune response to the streptococcal antigen (SACHO).

In the above studies, animals receiving anti-Id-1 neonatally were Id-1 suppressed, even though their initial exposure to GASV was not until 3 months of age. In an additional study we observed that significant suppression was still apparent if this time period was extended to 11 months. F344 rats neonatally injected with anti-Id-1 antiserum, produced 13±8 μg/ml of Id-1 following immunization with GASV at 11 months of age, while control animals produced 167±63 μg/ml of Id-1.

Analysis of idiotype suppression induced in adult animals. To determine if Id-1 production could be permanently altered by exogenous manipulations of adult animals, as observed above for neonatal animals, we injected adult HPR rats with anti-Id-1 antibodies. In our initial studies to identify the most effective route of injection, 1 ml of anti-Id-1 antiserum was injected either iv or ip into 2.5 month old HPR rats. Both groups of animals were immunized iv with GASV 2 weeks later. Id-1 specific suppression was induced in 4 of the 4 rats injected with anti-Id-1 ip, whereas the same antiserum injected iv induced suppression in 1 of 4 rats (Table 2). To determine the duration of the adult induced suppression, we repeated these studies with the exception that specifically purified anti-Id-1 antibodies were injected ip and GASV immunization was initiated on day 5 rather than day 18. Five of the 6 HPR rats treated with anti-Id-1 in this manner

Table 2. Id-1 production by rats injected as adults with anti-Id-1 antibodies

Strain	Adult injection	Id-1[d]	anti-SACHO	No. of animals
		(μg/ml)		Id-1 suppressed
HPR	2 ml anti-Id-1 serum[a] (ip)	8±5	1755±393	4/4
	2 ml anti-Id-1 serum (iv)	1006±485	2131±496	1/4
	controls: 2 ml n. rabbit serum (ip or iv)	3049±1460	930±3350	1/6
HPR	400 μg s.p. anti-Id-1[b]	77±68	4810±958	5/6
	controls: 400 μg n. rabbit IgG	5735±2244	5831±1999	1/6
August	2 ml anti-Id-1 serum[c]	278±120	n.d.	3/6
	2 ml of 10 x concentrated anti-Id-1 serum	26±9	n.d.	6/7
	controls: nothing	478±158	n.d.	1/6

[a] 1 ml of anti-Id-1 or normal rabbit serum was injected on days 1 and 3. The iv GASV immunization protocol was initiated on day 14.

[b] 200 μg of s.p. purified anti-Id-1 or normal rabbit IgG was injected on days 1 and 3. GASV immunization was initiated on day 6.

[c] 1 ml of the indicated anti-Id-1 serum was injected on days 1 and 17. GASV immunization was initiated on day 32. Control animals were immunized with GASV at the same time.

[d] Refer to Table 1 for methods and notations.

were Id-1 suppressed (Table 2). Similar to the neonatal induction of suppression, adult induced Id-1 suppression was maintained until the termination of the study at 1 year (Figure 2).

Anti-Id-1 antiserum, prepared against Id-1 of HPR origin, could also effectively suppress Id-1 production when injected into adult August rats (Table 2). It is apparent from this study, however, that a critical concentration of anti-Id-1 containing serum was required for the induction of suppression, for no significant supression was induced in animals injected with the neat antiserum preparation (Table 2). Regardless of the concentration of anti-Id-1 injected ip or iv into adult rats, enhancement of Id-1 production was never observed.

In addition to being able to induce idiotype suppression by injecting anti-Id-1 into adult rats, Id-1 production could be suppressed by immunizing rats with Id-1 positive molecules. When F344 rats were extensively immunized with Id-1 bearing IgG, $F(ab')_2$ fragments, H-chains or L-chains, a majority of the animals had suppressed production of Id-1 following their later immunization with GASV (Table 3). These results are particularly interesting since antibodies directed against Id-1 preferentially recognize H-chain associated determinants (Stankus and Leslie 1976b). Both chains, however, are required for the complete expression of Id-1.

We conclude from the above results that heterologous anti-Id-1 and the induction of Id-1 specific immunity in adult rats can serve as effective means of suppressing the production of Id-1.

Table 3. Id-1 production by F344 rats immunized with Id-1 bearing molecules

Pre GASV immunization[a]	Id-1	Anti-SACHO	No. of animals
	(μg/ml)		Id-1 suppressed
Id-1$^+$ IgG	99±50	416±160	3/5
Id-1$^+$ F(ab')$_2$	256±235	315±113	3/4
Id-1$^+$ L-chain	49±37	179±47	2/3
Id-1$^+$ H-chain	202±115	363±193	2/4
Controls	301±81	619±119	1/8

[a]In all experiments animals received 480 μg of the indicated Id-1$^+$ molecule over an 8-15 months immunization period. At the end of this period the GASV immunization protocol was initiated. Control animals received either saline in adjuvant following a comparable immunization protocol or nothing. Id-1 immunization began at 2.5 months of age.

[b]Id-1 and anti-SACHO quantified by RIA analysis

[c]An animal was defined as being Id-1 suppressed if it produced <30 μg/ml of Id-1 throughout the GASV immunization protocol.

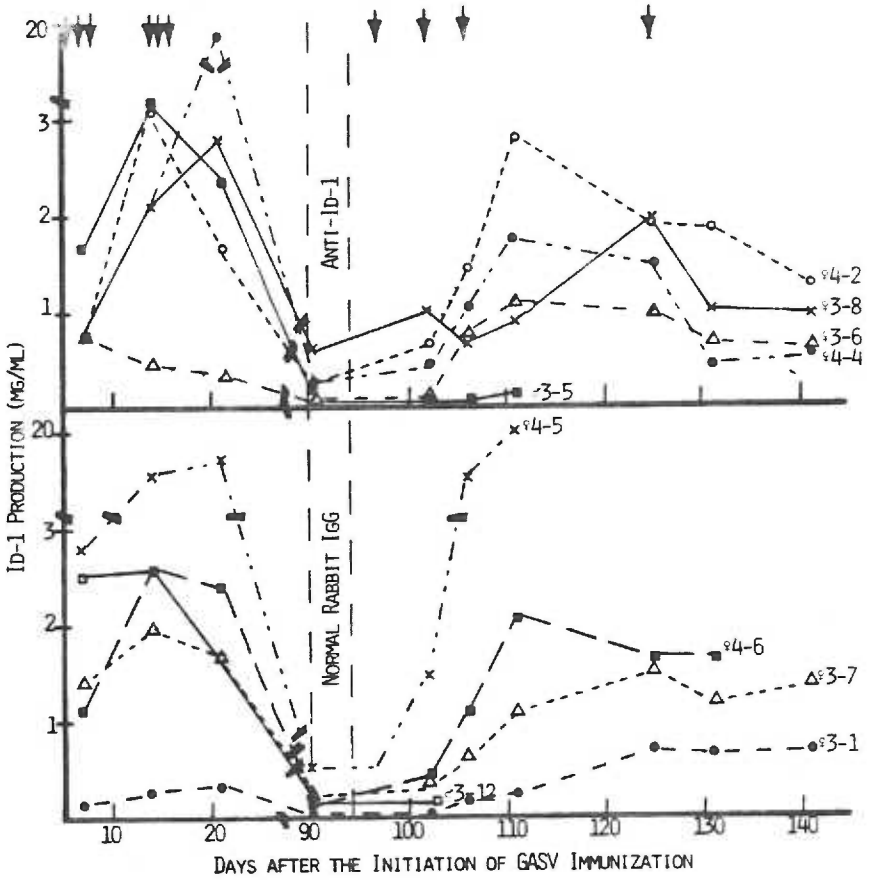
The effect of anti-Id-1 injections prior to secondary antigenic challenge. In the above studies Id-1 suppression was induced when animals were treated with anti-Id-1 prior to initial challenge with GASV. Treatment with anti-idiotypic antibodies before onset of disease, however, is usually not practical. Unfortunately, previous attempts at using anti-idiotypic reagents to suppress idiotype production after exposure to antigen have generally been unsuccessful (Beatty et al. 1976, Owen and Nisonoff 1978, Lanier et al. 1979, Brown et al. 1979) with the exception of the reports by Pawlak et al. (1974) who induced successful suppression of the cross-reactive idiotype in mice given anti-idiotypic antibodies 14 and 17 days after initial immunizations with azophenylarsonate. We wished to increase an understanding of the potential for anti-idiotypic antibodies to suppress idiotype production in a secondary immune response. Therefore, HPR rats, previously immunized with GASV and known to produce Id-1, were injected with anti-Id-1 antibodies just prior to rechallenge with antigen. In our initial study, animals were allowed to rest approximately 10 months after GASV immunization before anti-Id-1 injections were given. This interim period was reduced to 2.5 months in a second study. In both instances an IgG anti-Id-1 preparation having an antigen binding capacity of 100 µg was injected iv into each experimental rat, followed by the same quantity of anti-Id-1 given ip in two injections 3 and 5 days later. The secondary series of injections of GASV immunizations were given iv as indicated in Figure 3. The two routes of injection of anti-Id-1 were used because this allowed the antibody to encounter

antigen specific cells seen by the primary iv injections of GASV and also included ip exposure to anti-Id-1 which, as shown above, was more effective in suppressing Id-1 production in a primary response. The concentrations of anti-Id-1 injected were sufficient to suppress Id-1 production in a primary immune response. Control animals received normal rabbit gammaglobulin in place of anti-Id-1.

The kinetics of Id-1 production of the 10 HPR rats involved in one study are shown in Figure 3; results from both studies were comparable. It is apparent that treatment with anti-idiotypic antibodies, as specified by the above protocol, did not induce the Id-1 suppressed state obtained when anti-Id-1 was injected into adult rats prior to primary challenge with GASV. Two events, however, provide evidence that Id-1 specific immunity was developing within the anti-Id-1 treated rats. In one study the ratio of the concentrations of Id-1:Anti-SACHO in the serum 2 weeks after the initial injection of anti-Id-1 was much lower in the anti-Id-1 treated rats (0.85) than in control rats (3.59). This study was terminated at week 2 due to the poor viability of older rats upon challenge with GASV. In the study shown in Figure 3, we were able to monitor Id-1 production for a prolonged period of time. This permitted us to determine if anti-Id-1 required more time to alter an on-going immune response. After the last of several challenges with antigen on day 125, Id-1 production in all those animals receiving anti-Id-1 appeared to wane, whereas control animals maintained pre-existing Id-1 concentrations.

It is evident from these studies that the administration of anti-Id-1 antibodies to adult animals prior to secondary challenges with

Figure 3. The kinetics of Id-1 production by HPR rats receiving anti-Id-1 prior to secondary challenges with GASV. Animals in upper panel were injected iv with anti-Id-1 on day 90 and ip with anti-Id-1 on days 92 and 95. Animals in lower panel received normal rabbit gammaglobulin in place of anti-Id-1. Arrows indicate time of iv injections of 1 mg of GASV, with the exception of the last arrow, which represents the injection of 0.2 mg of GASV.


ID-1 PRODUCTION (MG/ML)
ANTI-ID-1
NORMAL RABBIT IGG
♀4-2
♀3-8
♀3-6
♀4-4
♀3-5
♀4-5
♀4-6
♀3-7
♀3-1
♀3-12
DAYS AFTER THE INITIATION OF GASV IMMUNIZATION

antigen does not effectively suppress Id-1 production, but there is suggestive evidence that this treatment may induce a weak idiotype specific immunity that becomes more effective with time.

Discussion

Before idiotypic and anti-idiotypic antibodies can be used clinically, it is essential to have a thorough understanding of the effects of these reagents when administered in vivo. This is particularly important in view of the evidence that different idiotypic systems appear to have different mechanisms of regulation (discussed in more detail below). A greater understanding of the effectiveness of idiotype specific manipulation in altering immune responsiveness can be obtained by performing such manipulations in animal models using a variety of idiotypes which reflect immunity to different antigens. The purpose of these studies was to analyze extensively the ability of exogenous manipulations to regulate the production of one such idiotype (Id-1) and compare this to results from other investigators who have undertaken similar studies. Similarities and differences between idiotypic systems might allow one to speculate rationally on the direction which idiotype specific manipulations should take with regard to their clinical application as effective immune modulating tools.

When Id-1 production was monitored relative to time, it became evident that its production could fluctuate independently of antigenic challenge and anti-SACHO antibody production. This suggested the involvement of autologous idiotype specific regulatory mechanisms in the

production of Id-1. The existence of functionally independent Id-1 specific regulation was further supported by the fact that Id-1 production could be manipulated by injecting anti-Id-1 antibodies in vivo without quantitatively altering the animal's potential to produce anti-SACHO antibodies. Intraperitoneal injection of anti-Id-1 antibodies to either neonatal rats or adult rats induced permanent Id-1 suppression, as monitored for a 9 month period, during which time intermittent re-challenges with GASV were given. This suppression could be induced in all tested strains of rats, regardless of major histocompatibility haplotype, or immunoglobulin allotypes, using a single reagent prepared against a restricted population of anti-SACHO antibodies obtained from one HPR rat.

It was previously shown that suppression of the cross reactive idiotype (CRI) associated with anti-phenylarsonate antibodies (Hart et al. 1972) and the A5A idiotype associated with Group A streptococcal carbohydrate antibodies (Hetzelberger and Eichmann 1978) could be induced by injecting adult A/J mice with the appropriate anti-idiotypic reagent. These idiotypes and Id-1 serve to identify determinants associated with only a fraction of the antibody molecules having specificity for the particular antigen. In contrast, it is more difficult to induce suppression of idiotypes associated with the majority of the antibody molecules of a given specificity. For example, in BALB/c mice the T15 idiotype on anti-phosphorylcholine antibodies and the IdX on anti-inulin antibodies cannot be permanently suppressed by administering the respective anti-idiotypic antibodies to adult animals (Strayer et al. 1975, Bona et al. 1979). Neonatal animals, however, are more

sensitive to anti-idiotypic treatments as evidenced by the successful induction of idiotype suppression in animals given anti-T15 or anti-Id antibodies neonatally. It is apparent, therefore, that one must study the feasibility of inducing idiotype suppression based both on the nature of the idiotype being suppressed and on the state of immunological maturity of the individual being treated.

It is evident from our results that the effectiveness of anti-idiotypic antibodies in inducing Id-1 suppression in adults can be increased depending upon its route of injection (ip injection of anti-Id-1 being more effective than iv). Id-1 suppression was induced if GASV immunization was initiated from 5 to 32 days after first exposure to anti-Id-1, suggesting that at least within these limitiations, the timing between anti-Id-1 and GASV injections was not critical. In addition, although a longer period of time may be required, idiotype suppression can also be induced by immunizing animals with Id-1^+ IgG, $F(ab')_2$ fragments, or H- or L-chains prior to GASV immunization. Interestingly, in this limited study, all immunogens proved to be equally effective in inducing suppression, even though Id-1 immunodominant determinants are predominantly H-chain associated (Stankus and Leslie 1976a).

As with the injection of adult animals with anti-idiotype, there are both advantages and limitations in manipulating immune responsiveness via neonatal administration of anti-idiotypic antibodies. The increased sensivity of the neonatal immune system, which allows the permanent redirection of immune responsiveness, is also associated with broadening the realms of the anti-idiotypic probe. For example,

although a majority of the animals injected ip with anti-Id-1 neonatally were Id-1 suppressed following GASV immunization at 3 months of age, repeatedly a sibling receiving the same anti-idiotypic preparation, rather than being suppressed, actually appeared to have enhanced Id-1 production. The enhancement of idiotype by anti-idiotypic antibodies in vitro is not a new observation, but previously this enhancement was only apparent when low concentrations of anti-idiotype were administered (i.e. 0.01 µg of anti-idiotype used by Hiernaux et al. 1980, or 0.28 µg of anti-idiotype used by Trenkner and Riblet 1975). Even with 200 µg of specifically purified anti-Id-1 antibody we did not increase the relative efficiency of suppression, nor did we eliminate the apparent enhancing influence of anti-Id-1. In contrast, enhancement of Id-1 was never observed when adult rats were injected ip or iv with anti-Id-1 preparations, regardless of the concentrations injected. These studies make us aware of two important points. First, the same reagent has the potential of inducing opposite influences in vivo when injected into animals having nearly identical genetic backgrounds, and second, the variable influences of the anti-idiotypic probe in our hands are apparent when administered to the immunologically immature neonatal animal but not adults. This increased sensitivity of neonatal animals to the variable effects of idiotypic manipulations is also evidenced by permanent idiotype specific alterations induced by maternal immune reactivity (Olson and Leslie 1981). Whether this difference is due to: a) an increased vulnerability of neonatal cells; b) accessibility to different cell types due to preferential sequestration of cells with

age; c) modifications associated with the extended time period between neonatal exposure to anti-Id-1 and immunization with antigen; or d) in situ concentrations of autologous idiotype specific immune factors remains to be determined. This latter possibility becomes increasingly relevant in view of our observation that the injection of Id-1 in conjunction with anti-Id-1 neonatally can lead either to significantly enhanced or suppressed Id-1 production depending upon the ratio of Id-1 to anti-Id-1 injected.

Although it is evident that neonatal as well as adult injections of anti-Id-1 have the potential to alter immune responsiveness, both of the previously mentioned approaches take into consideration that the individual has not been challenged with the specific antigen prior to anti-idiotype administration. In clinical situations today this order of treatment is not commonly practical. Rather, one is faced with treating a disease after an idiotype specific immune response has been initiated. There is precedent, however, for the effectiveness of autologously induced anti-idiotype immunity in altering the course of immunological disease and increasing graft survival (Abdou 1981, Miyajima et al. 1981), implying that if idiotype immunity could be manipulated after exposure to antigen, this would be advantageous to the individual.

Previously published studies showed that effective inhibition of idiotype specific immune reactivity could only be obtained if anti-idiotypic antibodies were administered before or very close to the time of antigenic exposure (Beatty et al. 1976, Owen and Nisonoff 1978, Lanier 1979, Brown et al. 1979). The increased resistance of a secondary immune response to anti-idiotype treatments was found to be related to the increased resistance of secondary B cells to suppression (Owen and

Nisonoff 1978). We found that animals receiving anti-Id-1 either 2.5 or 10 months after the primary GASV immunization protocol similarly had increased resistance to anti-Id-1 induced suppression. Decreased Id-1 to anti-SACHO ratios, and increased rate of decline of Id-1 in anti-Id-1 treated as compared to control animals, however, gave some indication of the development of an Id-1 specific immunity with time. Unfortunately, the poor survival of all the animals following secondary challenges with antigen, prohibited our monitoring the possible development of a significant Id-1 suppressive effect. Although it is evident from these studies that antigen primed animals did not obtain the idiotype suppressed state observed in unprimed animals, our results, in addition to those obtained by Zanetti and Bigazzi (1981) who observed more effective suppression of anti-rat thyroglobulin antibodies with increasing time after exposure to anti-idiotypic antibodies, lend a glimmer of hope for further investigations of the use of anti-idiotypic antibodies in the specific suppression of a secondary immune response.

In conclusion, the administration of anti-idiotypic antibodies in vivo has the potential to: a) totally redirect a specific immune response if injected neonatally; b) has the potential to suppress immune reactivity if injected into adults; and c) appears to have the potential to induce an idiotype specific immunity against a secondary immune response. In this latter instance, the immunity requires an extended period for induction and its effectiveness most likely does not approach that seen for a primary response. It is now apparent that anti-idiotypic antibodies that serve as effective regulatory tools can be prepared against homogeneous, restricted or heterogeneous immune responses

in animals and humans (Nough et al. 1976, Brown et al. 1980, Nagelkerken et al. 1980. In addition, as with the Id-1 idiotype, cross reactivities amongst idiotypes extend the regulatory capacities of a particular anti-idiotype reagent to affect not only closely related antibody populations within the same individual but also antibodies of the same specificity within different individuals of varying genetic backgrounds (Kunkel et al. 1973, Bellgrau and Wilson 1979, Forre et al. 1979, Andrews and Capra 1981, Fritz and Desjardin 1981, Vincent 1981). Regarding the clinical future of anti-idiotypic antibodies, there is no doubt that such reagents offer precision to immunological manipulations not offered by any yet identified immunoregulatory tool. When anti-idiotypic antibodies prepared against a non-homogeneous immune response and carefully defined as to class and subclass, are injected by an appropriate route into adults, one can be fairly confident that a degree of idiotype suppression will be induced. Although such suppression alone may not be adequate for disease control, complemented by other treatment, this may offer an additional specific advantage which allows the host to survive (Lanier et al. 1979).

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BRIEF COMMUNICATION

Manipulations analyzing the regulatory limits of the Id-1 immune network

Id-1 was found only on antibodies specific for SACHO and was not associated with anti-SACHO antibodies produced by any other species (Stankus and Leslie 1977). Studies by Urbain et al. (1977) and Cazenave (1977), however, showed that the production of a specific idiotype could be induced in rabbits, who normally do not produce the idiotype, if animals were immunized with an antibody against the idiotype prior to antigenic challenge. This is consistent with data from several laboratories which indicated that the immune repertoire expressed by an individual may not be a true reflection of the individual's genetic potential, but rather a reflection of regulatory influences exerted on gene expression (Bosma et al. 1978, Cancro et al. 1978, Bona and Paul 1979, Gearhart and Cebra 1979). Together these studies suggest that one might be able to broaden the immune repertoire expressed by an individual by biasing immunoregulatory mechanisms to favor the expression of desired clones.

1. Induction of Id-1 in association with chicken anti-SACHO antibodies

To enhance the probability that an Id-1 clone, if present in chickens would be stimulated by GASV, we first immunized chickens with rat Id-1 positive molecules, then challenged the animals with GASV. The protocol was as follows: 3 month old S/C chickens were injected subcutaneously, at multiple sites, with 100 µg of Id-1^+, rat anti-SACHO IgG or $F(ab')_2$ fragments in FCA. Two weeks later the birds were injected subcutaneously with 50 µg of the same Id-1^+ preparation in FIA. GASV immunizations began 1 month after the last injection of Id-1.

Control birds were immunized with the same amount of normal rat IgG, in place of the Id-1$^+$ preparation. An additional group of control chickens was immunized with 60 then 15 µg of chicken anti-SACHO antibodies prior to GASV challenges. Id-1 and anti-SACHO production of these birds were analyzed for 9 months. During this time periodic injections of GASV were given.

As shown in Figure 1, sera obtained from Id-1 immunized birds were capable of inhibiting the Id-1 RIA by as much as 50%. Sera from control animals, in contrast, were ineffective as inhibitors in the Id-1 RIA (maximum inhibition = +/-5%, Fig. 2). Anti-SACHO production by the experimental and control groups was comparable.

The kinetics of Id-1 production emphasized two points: 1. the inhibition of the Id-1 RIA was apparent in many of the animals prior to antigenic challenge, and 2. the production of the inhibitor could be affected by injections of antigen. To analyze the nature of these inhibitors, both before and after antigenic challenge, selected samples (as indicated by the circles around points on the Id-1 graphs in Figure 1) were passaged over:

1. An N-acetyl glucosamine (N-AcGln) immunoadsorbent (IA) to determine if the inhibitor was associated with anti-SACHO molecules.

2. An anti-Id-1 IA to verity that the inhibitor was Id-1 rather than anti-Id-1.

3. An anti-IgY IA to determine if the inhibitor was on chicken, rather than rat, immunoglobulin molecules.

Figure 1. Id-1 and anti-SACHO production by 6 chickens initially immunized with either Id-1^+ IgG (left graphs) or Id-1^+ $F(ab')_2$ fragments (right graphs). Solid lines represent Id-1 production, while dashed lines represent concentrations of precipitating anti-SACHO antibodies. Circled points on the Id-1 graph denote samples which were passaged over various IA columns in order to verify the nature of the reactive component. XX represent subcutaneous injections of 100 and 50 µg, respectively, of the indicated Id-1^+ protein. Arrows indicate injections of 1 mg of GASV iv.

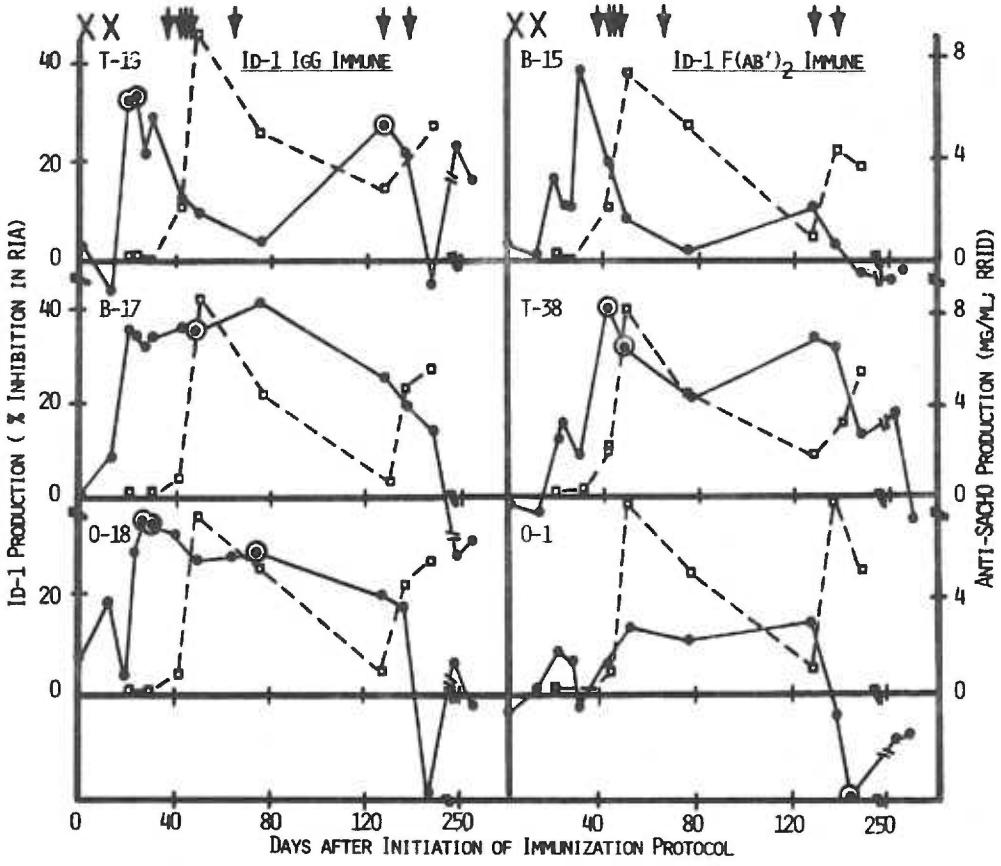
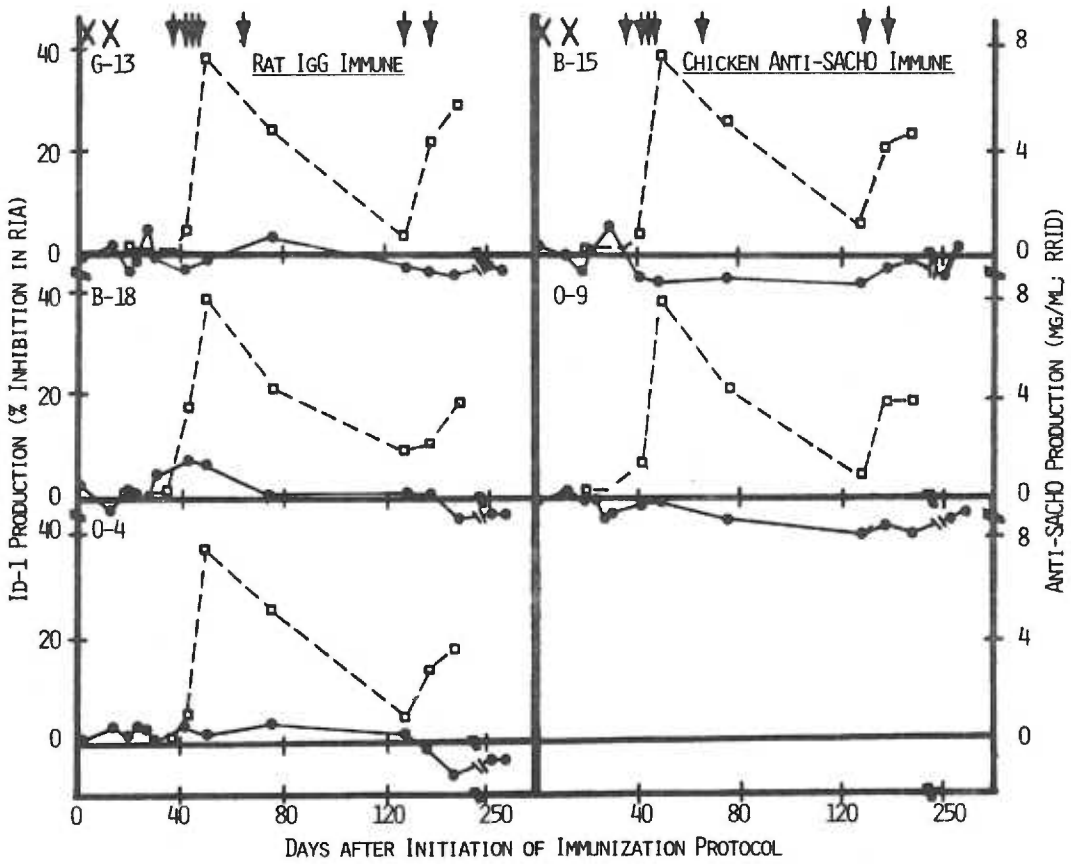

T-13
ID-1 IgG IMMUNE
B-15
ID-1 F(AB')2 IMMUNE
B-17
T-38
0-18
0-1
ID-1 PRODUCTION (% INHIBITION IN RIA)
ANTI-SACHO PRODUCTION (MG/ML RRID)
DAYS AFTER INITIATION OF IMMUNIZATION PROTOCOL
0
40
80
120
250
20
4
8

Figure 2. Id-1 and anti-SACHO production of rat IgG immune and chicken anti-SACHO immune control chickens. Refer to Figure 1 for graph details.


G-13
RAT IgG IMMUNE
B-15
CHICKEN ANTI-SACHO IMMUNE
B-18
O-9
O-4
ID-1 PRODUCTION (% INHIBITION IN RIA)
ANTI-SACHO PRODUCTION (MG/ML; RRID)
DAYS AFTER INITIATION OF IMMUNIZATION PROTOCOL
0
40
80
120
250
20
8
4

The results from 3 birds are shown in Table 1. All other sera tested gave negative results. Unless indicated, separate serum aliquots were applied to each column.

T-19, day 20-23 sera, were effective inhibitors in the Id-1 RIA, and the inhibiting component could be eluted from an anti-Id-1 IA column. This sample appeared to lack anti-Id-1 reactivity, for it was unable to bind to an Id-1^+ radiolabeled probe (data shown below). The Id-1 inhibitor in these sera could represent residual rat Id-1^+ protein. Further tests are in progress to determine this.

B-17 (day 50 serum) was also an effective inhibitor of the Id-1 RIA. This activity could be eluted from an N-AcGlN IA column, which verifies its SACHO specificity. T-17 sera obtained prior to challenge with GASV was also an effective inhibitor in the Id-1 RIA. However, this activity could not be eluted from the hapten IA column. This suggested that a low percentage of the anti-SACHO antibodies produced by this chicken, following immunization with GASV, actually possessed Id-1 idiotypic determinants.

To determine if these antibodies were of chicken, rather than rat origin, the Id-1^+ eluate off the N-AcGln column was passaged over an anti-chicken IgY IA column. As shown in Table 1 the majority of the inhibitor activity was eluted from the IgY immunoadsorbent. This further supports the chicken origin of this Id-1^+ anti-SACHO antibody.

The most unusual results in these studies were obtained from chicken O-1. Rather than inhibiting the Id-1 RIA, serum obtained on day 148 enhanced binding of the radiolabeled probe by >30%. This

suggested that the animal was producing anti-Id-1 antibodies. Passage of this serum over an N-AcGln IA showed that more of the enhancing activity was eluted from the column than remained in the effluent. (This effluent and eluate were also analyzed undiluted in the RIA to confirm the differences between samples.) No activity was absorbed by the anti-Id-1 IA. The eluate off the N-AcGln IA was analyzed for the presence of anti-Id-1 immune reactivity by its ability to bind an $Id\text{-}1^+$ radiolabeled probe. The data are shown below:

Chicken:	Preparation		% Bound to radiolabeled probe	
			$Id\text{-}1^+$ rat IgG	$Id\text{-}1^-$ rat IgG
0-1	eluate off N-AcGln IA	undil.	64.0	-1.1
		1:2	65.4	-1.1
		1:10	13.0	2.6
T-19	eluate off anti-Id-1	undil	0.9	-2.7
	IA (control)	1:2	4.2	-2.8
		1:10	5.9	0.5

The ability of the N-AcGln eluate produced by chicken 01 to bind radiolabeled Id-1 suggests that this preparation had specificity for both SACHO and Id-1. One interpretation of these data is that GASV immunization of this Id-1 primed chicken induced anti-SACHO antibody molecules with anti-Id-1 rather than Id-1 idiotypic determinants. It is also possible that the activity of two antibodies, in an Id-1 anti-Id-1 immune complex, is being detected in the assays. If this is so, it is highly unlikely that the rat Id-1 initially injected would be detectable in serum after 148 days, thus implying the chicken origin of both

Table 1. Analysis of sera from Id-1 immunized chickens

	Day after iniati-ation of pro-tocol	Serum			Over hapten (N-AcGln) IA							
					Effluent				Eluate			
		Id-1[a] % Inh	IgY[b] mg/ml	Id-1:[c] IgY	Id-1 % Inh	IgY mg/ml	Id-1: IgY	Anti-SACHO[d] mg/ml	Id-1 % Inh	IgY mg/ml	Id-1: IgY	Anti-SACHO
T-19	20-23	33.0	10.7	0.4								
	128	27.9	>11.0	0.38	12.9	10.6	0.17		5.7	1.15	<0.9	
B-17	50	34.4	>11.0	1.4	35.2	9.0	1.0	1.3	28.0	0.14	39.2	4.0
0-1	148	-34.0	12.0		-2.3 [undil. -24.1]	9.9	-		-6.7 [undil. -43.3]	3.6		

Table 1 (Continued)

	Day after initiation of protocol	Over anti-Id-1 IA: Effluent Id-1 % Inh	Effluent IgY mg/ml	Effluent Id-1:IgY	Eluate Id-1 % Inh	Eluate IgY mg/ml	Eluate Id-1:IgY	Over anti-IgY IA: Effluent Id-1 % Inh	Effluent IgY mg/ml	Effluent Anti-SACHO mg/ml	Eluate Id-1 % Inh	Eluate IgY mg/ml	Eluate Anti-SACHO mg/ml
T-19	20-23	20.6	6.0	0.5	19.4	0.09	30.0						
	128	16.3	9.3	0.25	0	0.18	0						
B-17	50							1.9	<0.09	<0.1	13.4	0.4	1.7
0-1	148	31.0	>11.0		1.9	<0.1							

[a]Id-1 quantified by RIA. All samples run at 1:10 dilution, unless indicated.

[b]IgY quantified by radial immunodiffusion.

[c]Id-1:IgY ratio x 10^{-3}.

[d]Anti-SACHO quantified by RRID.

antibodies in the complex, if it exists. Regardless of the nature of this Id-1 binding protein, the fact that it is first detectable 130 days after immunization with rat Id-1, but only 10 days after a re-challenge with GASV, emphasizes the influential role of GASV in the induction of such Id-1 specific immunity.

It can be concluded that immunization of chickens with Id-1 prior to challenge with antigen, alters the clonotype of the immune responsive to GASV. Specifically anti-SACHO antibodies bearing either idiotypic or anti-idiotypic determinants appear to be induced in a species previously not known to produce Id-1.

Studies are in progress to determine if the Id-1 or anti-Id-1 immune reactive components in chicken B-17 and O-1 represent immune complexes, or specific antibody molecules.

2. Induction of Id-1 in association with rat antibodies of non-SACHO specificity.

To determine if Id-1 could be associated with antibodies specific for non-SACHO antigenic determinants, the Id-1 immune network was stimulated by injection of Id-1 prior to immunization with the non-SACHO antigen. Two antigens were employed in these studies. Both were selected because they might favor the induction of Id-1 bearing antibodies.

Sheep red blood cells (SRBC) is an antigen with multiple antigenic determinants. Although Id-1 was not previously found to be associated with rat anti-SRBC antibodies, a possible weak cross-reactivity between SACHO and one of the determinants on SRBC might induce Id-1^{+}

anti-SRBC antibodies, provided that the Id-1 immune network was stimulated prior to challenge with GASV. Similarly, immunization of mice with bacterial levan (a β 2-6 polyfructosan with β 2-1 branch points) has been shown to induce the production of antibody molecules which recognize SACHO in addition to those with specificity for levan (Enghofer personal communication). Initial studies in rats indicated that levan immunization may stimulate Id-1 production in association with a small percentage of the anti-levan antibodies (Fig. 3).

F344 rats were injected ip neonatally with 16 μg of Id-1$^+$ IgG in combination with 80 μg of anti-Id-1. Control rats received 16 μg of normal rat IgG and 80 μg of normal rabbit IgG. At 3 months of age all rats were immunized iv with 1 ml of a 10% SRBC solution, two times per week for three weeks. The Id-1anti-Id-1 complex was used to prime Id-1 immunity because previous studies showed that this treatment may enhance GASV induced Id-1 production (Olson and Leslie in preparation).

As shown in Figure 4, the ability of neonatally Id-1 primed, SRBC immune rats to inhibit binding in the Id-1 RIA was not significantly altered from that of control animals. Similar results were obtained from studies in HPR rats. The HPR rats received 100 μg of Id-1 ip neonatally and were immunized with 10% SRBC iv via the standard GASV immunization protocol at 3 months of age. Control animals received 100 μg of normal rat IgG in place of Id-1. Throughout the 3 week immunization period Id-1 production by both Id-1 injected and control animals remained negligible (<3 μg/ml). Experimental and control animals in these studies produced comparable concentrations of anti-SRBC antibodies.

Figure 3. Id-1 production by Copenhagen rats immunized with bacterial levan. Arrows indicate the injection of 15 μg of levan ip.

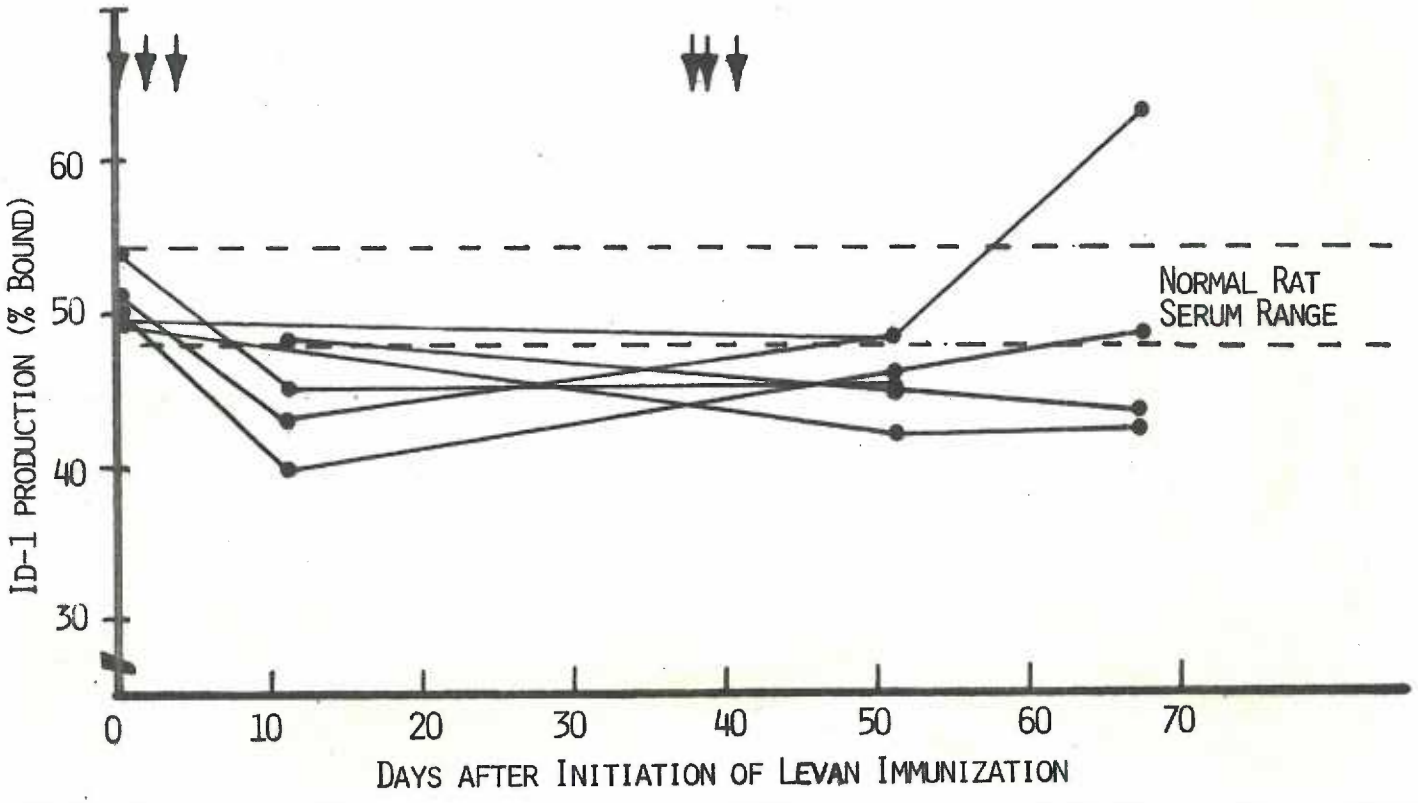
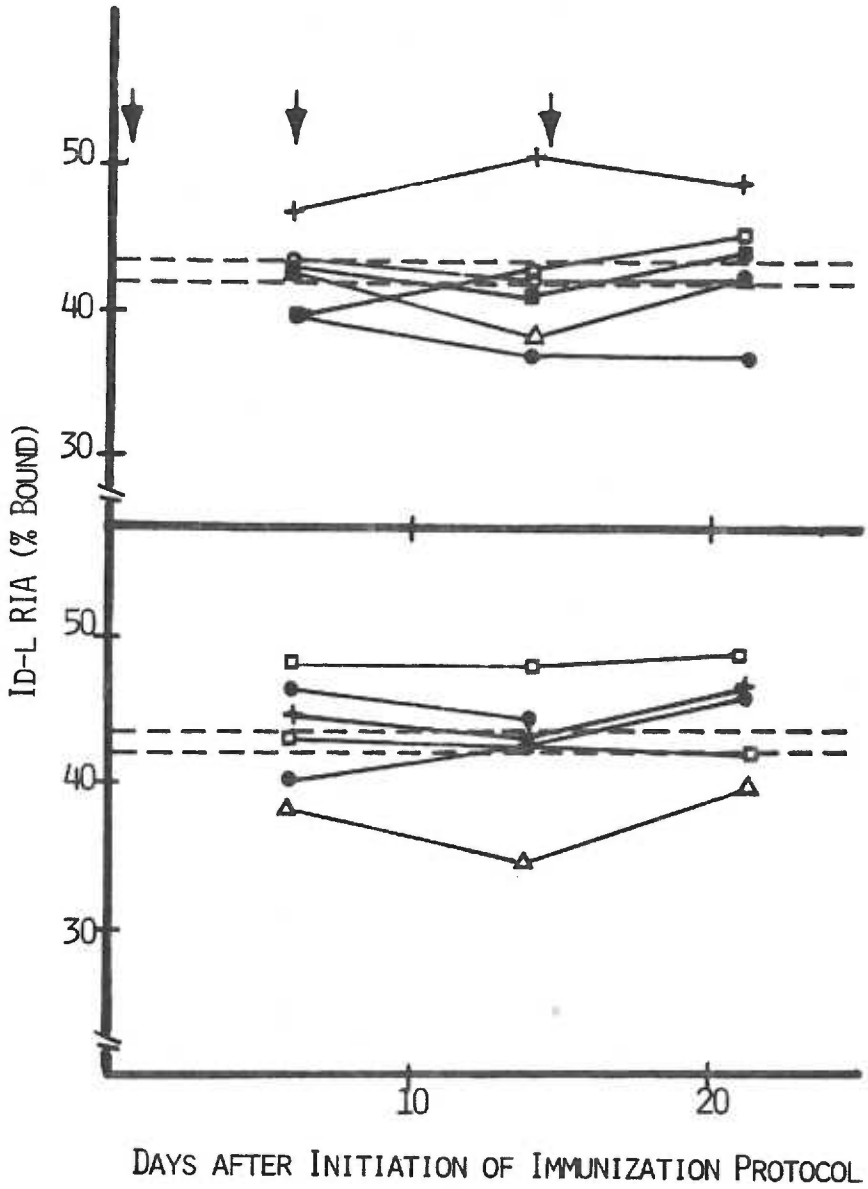

ID-1 PRODUCTION (% BOUND)
60
50
40
30
0
10
20
30
40
50
60
70
DAYS AFTER INITIATION OF LEVAN IMMUNIZATION
NORMAL RAT
SERUM RANGE

Figure 4. Id-1 production of SRBC immunized F344 rats neonatally injected with 80 μg of anti-Id-1 and 16 μg of Id-1 (upper graph) or 80 μg of rabbit IgG and 16 μg of rat IgG (lower graph). At 3 months of age, animals were injected iv with a 10% SRBC suspension. Arrows represent each injection of 1 ml of the SRBC suspension.


50
40
30
50
40
30
10
20
ID-L RIA (% BOUND)
DAYS AFTER INITIATION OF IMMUNIZATION PROTOCOL

To examine the induction of Id-1 in association with anti-levan antibodies, adult F344 rats were injected subcutaneously with 50 μg of Id-1 in FCA. Control animals received 50 μg of normal rat IgG in FCA. One week later all animals were immunized with 15 μg of levan ip. This same injection was repeated 3 and 7 days later. Id-1 analysis of immune sera obtained during this period from both experimental and control groups showed no significant difference, nor any induction of Id-1.

It is evident that neonatal or adult exposure to Id-1 as described in the above studies did not induce the association of Id-1 with antibodies of different specificities. I later showed, however, that the methods used did not consistently induce enhanced Id-1 production following GASV immunization. The most effective means of inducing Id-1 enhancement was to inject neonatal rats with Id-1 conjugated thymocytes. Before it can be concluded that idiotypic manipulations were unable to induce Id-1 in association with antibodies of different specificities, it is essential that this more effective means of stimulating Id-1 production be analyzed.

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SUMMARY AND CONCLUSIONS

Id-1, a cross-reactive idiotype associated with rat anti-Group A streptococcal carbohydrate (anti-SACHO) antibodies, was used to extensively analyze the potential of idiotype specific regulatory mechanisms to influence immune responsiveness in vivo. The importance of endogenous idiotype-specific regulatory mechanisms in altering an individual's immune response following antigenic challenge were confirmed by maternally induced immunoregulatory influences on immune reactivity of progeny. Autologous Id-1 specific immunity of mother rats had the potential to induce significant alterations in the ability of her progeny to produce Id-1, following immunization of progeny with the streptococcal antigen (GASV) at 3 months of age. The non-genetic origin of these maternal influences was confirmed by foster mother studies. Alterations in total anti-SACHO production did not necessarily accompany Id-1 alterations, emphasizing the sometimes subtle nature of the maternal influences. Neonatal exposure to either Id-1 specific cells or antibodies or antigen could alter the potential of the individual to produce Id-1. During gestation and the neonatal developmental period, cells, antibody and antigen of maternal origin all have some degree of accessibility to the progeny, suggesting that the exact influence induced by the mother may result from a combination of influences induced by multiple maternally derived factors.

To analyze the potential of exogeneous idiotype specific manipulations to predictably alter immune responsiveness, rats were injected

neonatally and as adults with different idiotypic probes, at varying concentrations. The injection of heterologous anti-Id-1 antibodies could induce long-term Id-1 suppression, regardless of the age at which the antibody was given. Occasionally, however, an individual receiving anti-Id-1 neonatally, rather than being Id-1 suppressed, actually had enhanced levels of Id-1 production. Id-1 production was found to be most consistently enhanced by neonatal exposure to Id-1 conjugated lymphocytes, while neonatal exposure to Id-1 alone was less effective in enhancing Id-1 production. In contrast, similar exogeneous Id-1 specific manipulations of adult rats were never associated with enhanced Id-1 production. These results indicated that although suppressive mechanisms could be activated at any age, factors associated with the enhancement of Id-1 production were more vulnerable or accessible to manipulations in neonates.

It is now apparent from studies of several investigators that constraints on an individual's potential to produce a specific clone of antibody molecules may not be related to the presence or absence of the necessary structural genes, but rather to specific regulatory mechanisms the individual has adapted. To determine if Id-1 specific manipulations had the potential to redirect an individual's expressed immune repertoire, Id-1 immunity was induced: a) prior to challenge of rats with a non-streptococcal antigen and b) prior to challenge of chickens with GASV. (Id-1 is not naturally associated with anti-SACHO antibodies of chickens, or any tested species, other than the rat.) Although the idiotypic manipulations performed were not sufficient to induce Id-1 production in

association with rat immune responsiveness to a non-streptococcal antigen, it was apparent that the induction of idiotypic immunity prior to challenge of chickens with GASV could alter the clonal nature of chicken's immune response to GASV. Awareness of manipulations which lead to the selective expansion or suppression of immune reactive clones allows one to maximize the effectiveness of an individual's immune repertoir.

The complexity of idiotypic interactions, continually apparent in these studies, reflects the immune system's attempt to re-equilibrate idiotypic, anti-idiotypic and cross-reactive idiotypic interactions in vivo following perturbations of this network by antigen or one of these antibodies. An increased understanding of the advantages and limitations of idiotypic manipulations will allow such probes to be used clinically, where they can offer specificity to treatment and the diagnosis of disease previously not available. In this regard the subtle but significant idiotypic influences induced by maternal idiotype specific immunity may serve to explain the acquisition of hypersensitivities and autoimmune reactivities which appear to be inherited but in an unpredictable pattern.

Although the primary advantage of idiotypic manipulations is their precision, one also has the option to expand the diversity of such manipulations. Intraspecies cross-reactivities between idiotypes can extend the regulatory potential of idiotypic probes to different individuals. Hybridoma technology, in contrast, which now allows the preparation of monospecific human anti-human idiotypic antibodies, can

be used to increase the precision and consequently the predictability of idiotypic manipulations. The studies presented in this thesis extend our understanding of the potential for idiotypic interactions to regulate immune responsiveness in vivo and hopefully will facilitate their effective adaptation to clinical use.

APPENDIX 1

Analysis of rat and mouse anti-SACHO antibodies for shared idiotype determinants

Id-1 production by mice. Although Id-1 was associated with anti-SACHO antibodies produced by the 11 strains of rats which have been tested (Stankus and Leslie 1976), anti-SACHO antibodies produced by chickens, sharks, goats and rabbits did not possess Id-1 (Stankus and Leslie 1977). One species which was not previously analyzed for Id-1 production was the mouse.

Rat-mouse interspecies cross-reactive idiotypes have been identified by Sy et al. (1978). The idiotype CGAT associated with mouse anti-poly ($Glu^{60}Ala^{30}Tyr^{10}$) (anti-GAT) antibodies was identified on anti-GAT antibodies produced by LEW and F344 rats. The purpose of this study was to determine if anti-SACHO antibodies from rats and mice shared cross-reactive idiotypes.

Ascitic fluid from GASV immunized mice was kindly supplied to us by Dr. Moon Nahm (Washington Univ., St. Louis, MO). Each sample was analyzed for its Id-1 content in duplicate in the Id-1 competitive inhibition RIA. As shown in Table 1, 1:10 dilutions of mouse ascitic fluid samples containing as high as 10 mg/ml of anti-SACHO antibodies were unable to significantly inhibit binding in the Id-1 RIA. Rat ascitic fluid, in contrast, whose antibody content closely paralleled that of serum, could serve as a rich source of Id-1 (see Appendix 2).

Thus, although the mouse has been added to the list of tested species, Id-1 still remains unique to rat anti-SACHO antibodies.

The reciprocal study was also performed. Anti-SACHO containing sera obtained from different strains of rats were sent to Dr. Nahm for analysis of the cross-reactive idiotype associated with mouse anti-SACHO

Table 1. Analysis of Id-1 content of mouse anti-SACHO antibodies

Mouse ascites	Id-1 RIA (% Bound)[a]	Anti-SACHO[b] (mg/ml)	Ig class or subclass
Anti-SACHO ascites			
#1	54.4/52.6	3.9	IgG_3
#2	56.4/53.7		
#8	56.5/54.7	4.0	IgG_3
#11	53.6/53.1	9.8	IgG_3
#13	52.7/53.8		
#14	57.8/56.6		
#19	54.4/54.6		
#20	53.6/52.2		
#22	51.5/53.1		
#23	55.1/54.7	1.3	IgM
#30	51.7/	2.9	
Control ascites			
J606 myeloma	49.4/50.4/48.4		IgG_3 (k)
8-6A αDNP hybridoma	52.1/51.3/52.6		IgG_3

[a]% bound in Id-1 RIA was determined at a 1:10 dilution of the ascitic fluid. The results from the sample analyzed in duplicate are indicated.

[b]Anti-SACHO was quantified by RIA analysis.

(or anti-GAC) antibodies. The idiotype assay was performed in polyvinyl microtiter plates using immobilized N5 antibody (rabbit antibody to a hybridoma - HGAC-1) and HGAC8 as the radiolabeled proband. Different unlabeled inhibitors were then put into wells and titered out with 4X serial dilution. All the samples were run as duplicates. The negative control hybridoma is HGAC39 which has the same isotype (IgG3, K) as HGAC8. A 0.02 mg/ml concentration of HGAC8 inhibited the RIA by 50%. Unlike Id-1, this idiotype is primarily light chain associated, although the tertary structure established by the whole immunoglobulin molecule is essential for its complete expression (Moon Nahm, personal communication).

A total of 46 different sera from HPR, Copenhagen, August, Lou/Mn and (Copenhagen x HPR)F_1 hybrid rats were analyzed for the presence of the mouse GAC idiotype. The results of this study are shown in Table 2.

At lower dilutions of normal rat serum (1:2-1:8) a high non-specific inhibition in the idiotype RIA was observed (30-40%). Fourteen of the immune sera were able to reproducibly increase this inhibition; however, the highest inhibition observed was 50%.

It is evident that the mouse anti-GAC idiotype is not associated with a major portion of rat anti-SACHO antibodies. The small percentage of the molecules bearing the GAC idiotype does, however, appear to be real. The exact nature of these molecules must be examined before it can be concluded that rat anti-SACHO antibodies bear the mouse GAC idiotype.

Table 2. Analysis of mouse anti-SACHO idiotype in sera from GASV immunized rats

Strain	No.	Treatment	Anti-SACHO[b] (mg/ml)	Rat Id-1 Idiotype (mg/ml)	Mouse Idiotype (% Inh)
HPR		non-immune	n.d.	n.d.	<[c]
		non-immune	n.d.	n.d.	<
	3-6	GASV immune[a]	1.2	0	50
	1-8	" "	2.2	0	<
	6-3	" "	2.7	0	41
	6-5	" "	3.5	0	42
	3-2	" "	3.5	0	<
	3-3	" "	3.9	0	50/49
	6-1	" "	6.3	0	45
	5-2	" "	0.3	0.01	<
	4-2	" "	3.4	0.01	50
	5-6	" "	5.6	0.01	<
	1-5	" "	3.5	0.02	<
	4-3	" "	2.2	0.03	<
	4-4	" "	14.0	0.03	<
	1-1	" "	0.5	0.04	<
	4-4	" "	2.7	2.1	<
	3-1	" "	8.9	2.2	<
	2-4	" "	1.3	2.4	<
	6-2	" "	4.5	4.6	48
	5-3	" "	0	5.4	<
	2-6	" "	2.9	7.5	<
	2-4	" "	>20.0	>7.6	50/41
	2-7	" "	>20.0	>7.5	<
	1-2	" "	5.5	10.0	<
	3-2	" "	4.4	13.5	42
	5-3	" "	46.0	17.2	<

Table 2 - Continued

Strain	No.	Treatment	Anti-SACHO (mg/ml)	Rat Id-1 Idiotype (mg/ml)	Mouse Idiotype (% Inh)
Copen-hagen	3-6	non-immune	n.d.	n.d.	<
		"	n.d.	n.d.	<
	3-7	GASV immune	0	<0.01	42
	3-2	" "	0	2.7	<
	3-3	" "	1.2	6.2	<
	3-1	" "	0	6.5	45
	3-5	" "	2.5	6.8	41
Copen-hagen x HPR	11-1	non-immune	n.d.	n.d.	<
	11-10	"	n.d.	n.d.	<
	11-10	GASV immune	4.5	0.04	<
	11-1	" "	5.5	0.05	<
	11-9	" "	3.0	0.06	<
	11-13	" "	2.1	1.3	45
	11-3	" "	2.0	2.7	<
	11-8	" "	3.7	3.8	48
August		GASV immune	5.6	n.d.	<
Lou/mn		GASV immune	19.0	n.d.	<
F344	13-3	GASV immune	7.0	8.5	<
	13-4	" "	6.7	0.5	<

Table 2 - Continued

[a]Rats in this study received GASV iv via the standard immunization protocol. Sera from the second or third week of immunization are represented.

[b]Concentrations of precipitating anti-SACHO antibodies were determined by RRID. Id-1 and mouse anti-SACHO idiotype were quantified by RIA.

[c]Non-immune sera at a 1:8 dilution inhibited the RIA less than 40%. All sera samples were designated "<" if at a 1:8 dilution they inhibited the RIA $\leq$ non-immune sera. Where two numbers are indicated, the same sample was analyzed in two independent assays.

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APPENDIX 2

Utilization of rat ascitic fluid for monitoring immune regulation in vivo

Abstract

Rat ascitic fluid (ASF) obtainable in volumes up to 100 ml per tap, served as an efficient source of large amounts of antibodies and serum components. By all criteria tested, ASF protein components closely paralleled those found in sera. Female rats consistently produced approximately twice the volume of ASF as males; however, the quantity produced by each sex is strain dependent. We have sucessfully used ASF induction to increase the amounts of anti-carbohydrate, anti-hapten, anti-allotypic and anti-idiotypic antibodies produced from individual rats. In addition, due to the cellular nature of ASF, it serves as a convenient source of large numbers of leukocytes which can be obtained at concentrations up to $8x10^6$ cells/ml without sacrificing the animal. Rat ASF production decreased as the amount of fibrotic tissue within the peritoneal cavity increased. It appeared that this fibrosis, or another factor associated with ASF production, limited the reproductive capacity of female rats. The potential of using ASF as a source of both cellular and humoral immune reactivity to monitor regulation of immune responsiveness in vivo is discussed.

Introduction

One of the major difficulties in analyzing the in vivo regulation of immune responsiveness of most rodents is the inability to obtain sufficient quantities of lymphocytes and humoral immune factors from the same individuals to allow monitoring of immunological reactivity over an extended period of time. In 1957, Munoz reported that one

could increase the quantity of antibodies obtained from individual mice by injecting animals intraperitoneally (ip) with antigen in Freund's adjuvant. This treatment induced large volumes of peritoneal fluid which contained high concentrations of antibodies, specific for the injected antigen. Yaron et al. (1977), using a similar approach, induced the production of peritoneal fluid in guinea pigs and found purified antibodies and cells within this fluid to have the same specificity as that of lymph node cells and serum antibody. By adapting the method of Tung et al. (1976) to induce ascitic fluid (ASF) production in rats, we examined ASF for its potential to serve as an effective source of cellular and humoral immune factors. Parameters of analysis included: 1) comparison to serum composition; 2) immunoglobulin class and subclass distributions; 3) antibody content; 4) cellular composition; 5) duration of production; and 6) factors maximizing production.

The induction of ascitic fluid allowed us to obtain large amounts of antibody at different times during an immune response. Since the composition of serum and ascites was proportionately very similar, we were able to use antibodies purified from ascites to monitor in vivo modulation of specific antibody production with time. Such an approach can be used to obtain the large amounts of antibodies required for comprehensive analysis of the regulation of idiotype production, allotype production, and the regulation of the complementary anti-idiotype and anti-allotype production. In addition, the large number of lymphoid cells present within rat ascitic fluid permits parallel analysis of regulation of immune responsiveness at the cellular level.

Materials and Methods

Animals. The original breeding stock of Copenhagen (Cop), August (Aug), and Wistar Furth (W/F) rats were obtained through the courtesy of Clarence Reeder at the Veterinary Resources Branch of NIH, Bethesda, Maryland. Outbred Sprague-Dawley (SD) rats were originally obtained from a colony at Tulane University Medical School. Animals were subsequently bred or intra-bred within our laboratory.

Antigens. Group A streptococcus strain J17A4 was used to prepare Group A streptococcal vaccine (GASV) as has been previously described (Stankus and Leslie 1975). Cyanogen bromide (CNBr: Eastman, Rochester, N.Y.) activated GASV was used to couple GASV to ε-DNP-lysine (DNP: Sigma, St. Louis, Mo.). Briefly 100 mg (dry weight) GASV was suspended in 5 ml of water and adjusted to pH 11 with NaOH. Upon addition of 10 ml CNBr (1 gm/10 ml water) the pH was quickly adjusted and maintained at ∿10.5-11.5 with 1 NaOH until the pH stabilized (∿15 minutes). The mixture was centrifuged in the cold at 3,000 rpm for 20 minutes, washed with 50 ml of 0.5 M $NaHCO_3$ (pH 9), resuspended in 10 ml $NaHCO_3$ containing 20 mg DNP and stirred at 4 C overnight. The GASV-DNP was then washed twice with Tris buffer 0.01 M Trizma (Sigma, St. Louis, Mo.), 0.1% NaN_3 (Eastman, Rochester, N.Y.), 0.001 M EDTA (J.T. Baker, Phillipsburgh, N.J.), 0.14 M NaCl, adjusted to pH 7.4 .

Immunization. GASV primed hybrid rats were immunized as previously described (Stankus and Leslie 1975). Adult F344 rats were primed by immunization with GASV injected intraperitoneally (ip) three times a week for two weeks. Unless indicated, 1 mg of GASV or GASV-DNP was included in the antigen-adjuvant emulsion for ASF production.

Induction of ascites. Large volumes of ASF can be induced in rats with repeated ip injections of 1 ml of an emulsion consisting of a 9:1 ratio of Freund's complete adjuvant (FCA: Difco, Detroit, Mi.) to antigen or saline. Primed or unprimed rats of different strains were injected initially with a 9:1 ratio of Freund's incomplete adjuvant (FIA: Difco, Detroit, Mi.) to antigen. The purpose of the initial inoculation in FIA was to minimize ascites accumulation while increasing serum antibody levels (Tung et al. 1976). Two weeks after the initial inoculation of emulsion, rats were injected ip with an emulsion consisting of a 9:1 ratio of FCA to antigen. These injections were repeated weekly for 4 to 8 weeks. As ASF accumulated it was collected with an 18-gauge, 1.5 inch hypodermic needle, either with or without a syringe. For some experiments heparin (Panheparin, Abbott Laboratories, North Chicao, Il.) was mixed with ascitic fluid (1 unit/ml) to minimize clotting. Samples were centrifuged at 2000 rpm for 20 minutes and assayed for protein and antibody content. Antibodies were concentrated from large pools of ascitic fluid by precipitation with half-saturated ammonium sulfate $(NH_4)_2SO_4$ (Mallinckrodt, St. Louis, Mo.) .

Comparative analysis of serum and ascitic fluid.

a. Protein content. The total protein content of peritoneal ascitic fluid and serum was determined by measuring their refractive index with an Abbe' refractometer (Bausch-Lomb, Rochester, N.Y.). Protein composition was analyzed using cellulose acetate electrophoresis (Beckman Instruments, Inc., Fullerton, Ca.). The stained cellulose acetate membranes were scanned using a Quick Scan densitometer (Helena Laboratories, Beaumont, Texas). Immunoelectrophoresis (IE)

and Ouchterlony analyses were performed as previously described (Leslie and Clem 1969, Leslie and Hattier 1974).

b. Quantification of immunoglobulin classes and subclasses. The concentration of rat IgM, IgG_{2a}, IgG_{2c} and IgA were determined by radial immunodiffusion (Leslie and Swate 1972). Rabbit anti-rat IgM was prepared against specifically purified IgM anti-SACHO antibodies as previously described (Stankus and Leslie 1974, Ruddick and Leslie 1977). Anti-rat IgA and rat IgG_{2a} and rat IgG_{2c} myeloma proteins were kindly provided by Dr. Herve Bazin. Antisera to rat IgG_{2a} was prepared in rabbits by initially immunizing with rat IgG_{2a} myeloma protein (no. 1R418) purified as previously described (Bazin et al. 1974). Rabbits were later hyperimmunized with IgG_{2a} isolated from a normal rat serum pool by diethylaminoethyl cellulose (DEAE) ion exchange chromatography and elution with 0.005 M phosphate buffer, pH 7.5. Anti-IgG_{2a} was made γ 2a specific by passage through appropriate immunoadsorbent columns. Anti-IgG_{2c} was prepared in goats and guinea pigs immunized with specifically purified rat anti-SACHO antibodies of restricted heterogeneity (Stankus and Leslie 1976). The antisera were made γ 2c specific by passage over an IgG immunoadsorbent lacking IgG_{2c}. The purified anti-2a and anti-γ 2c were monospecific when tested by immunoelectrophoresis and Ouchterlony analysis against normal rat serum. The anti-γ 2c antibody gave a reaction of identity with anti-γ 2c antisera prepared in rabbits immunized with $IgG2_c$ myeloma protein (no. 1R3304).

c. Quantification of antibody concentrations. Anti-streptococcal carbohydrate (anti-SACHO) antibodies were assayed by radioimmunoassay (RIA) using a modified Farr technique as previously described (Olson

and Leslie 1981). Anti-dinitrophenyl (anti-DNP) antibodies were quantified by reverse radial immunodiffusion (RRID) in which 10 μg/ml of DNP-bovine serum albumin (BSA) was incorporated into an agarose gel employing "low salt" buffer (Leslie and Hattier 1974). Rabbit anti-DNP antibody, quantified byprecipitin analysis, was used as the reference standard in the assay. Id-1, a public idiotype associated with rat anti-SACHO antibodies (Stankus and Leslie 1974), was quantified by competitive inhibition RIA using rabbit anti-Id-1 (Olson and Leslie 1981).

d. Purification of anti-SACHO antibodies. To specifically purify anti-SACHO antibodies, sera or ascitic fluid was passaged over an N-acetyl glucosamine immunoadsorbent column (Stankus and Leslie 1976).

Cellular composition of ascitic fluid. The cellular composition of ASF was determined by morphological enumeration of ascitic fluid samples. Slides were prepared by cytocentrifugation (Cytospin Shandon Elliot, Surrey, England) 0.1 ml of diluted ASF at 1,000 rpm for 5 minutes and stained with Wright's stain through the kindness of Dr. K. Sheth (Clinical Hematology, University of Oregon Health Sciences Center).

Results

Comparison of ascitic fluid to serum. To determine if mechanisms regulating immune reactivity of serum co-regulate immune responsiveness within ASF, the composition of serum and ASF obtained from individual rats was compared. Cellulose acetate electrophoretograms of 8 paired samples obtained from GASV hyperimmunized rats showed no significant

qualitative differences between sera and ascites. Densitometeric readings of electrophoretograms of ASF and serum from two such GASV immunized animals are shown in Figure 1. Similarities in protein content of ASF and serum were confirmed by immunoelectrophoresis. When anti-SACHO antibodies were specifically purified from ASF and sera of a single animal by passage over an N-acetylglucosamine immunoabsorbent and sequential elution with: 1) temperature shift (from 4 C to 21 C), 2) 1 N sodium thiocyanate (NaSCN), and 3) 2 N NaSCN, each eluate from sera showed reactions of identity with the respective eluates from ASF when compared by Ouchterlony analysis against anti-normal rat serum anti-Id-1 antisera. Id-1 is a public idiotype associated with rat anti-SACHO antibodies (Stankus and Leslie 1974) .

Although qualitatively very similar, quantitative differences in ASF and serum content were evident. When total protein content of ASF and sera were compared, it was found that the amount of protein in ascites represented only about 63% of that found in serum (Table 1). Quantitative measurements of IgM, IgG_{2a}, IgG_{2c}, IgA and specific anti-SACHO antibodies in hybrid rats, and anti-hapten (anti-DNP) antibodies in F344 rats also reflected the overall decrease in protein content of ASF (Table 1). It should be mentioned, however, that although the concentration of antibody present in ASF was below that of serum, due to the large volumes of ascites routinely collected, the total amount of antibody obtained from ASF of individuals was increased an average of 22 times over that of animals repeatedly bled.

To further compare regulation of antibody production in ASF and serum, we monitored the concentrations of anti-SACHO and Id-1 antibodies

Figure 1. Densitometric readings of cellulose acetate electrophoretograms of serum from a non-immune hybrid rat, and paired ASF and serum from individual hybrid rats sampled at the same time (Serum ————; Ascites --------).

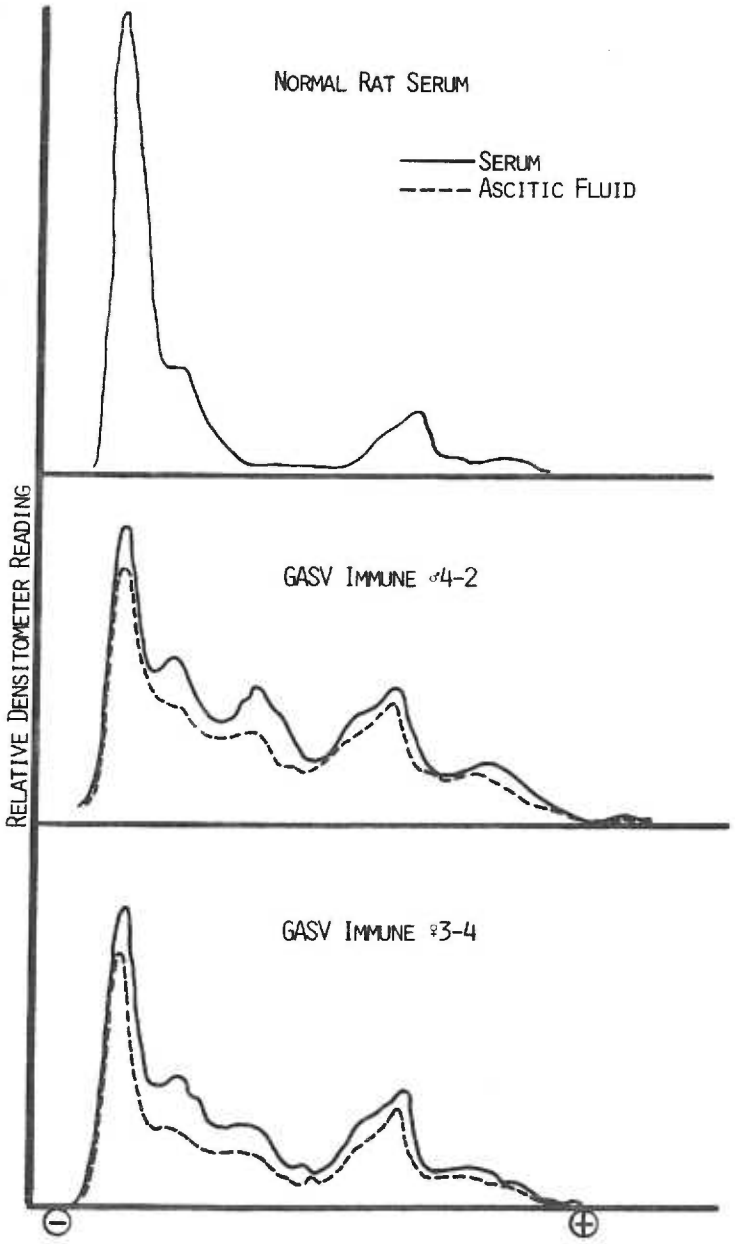
NORMAL RAT SERUM
SERUM
ASCITIC FLUID
RELATIVE DENSITOMETER READING
GASV IMMUNE ♂4-2
GASV IMMUNE ♀3-4
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Table 1. Quantification of proteins in rat ascitic fluid and serum

	Serum	Ascitic fluid	Ratio
	(mg/ml)		Ascites:Serum
Total Protein	62.9±1.4[a]	39.7±2.0	0.63±0.02
IgM	2.2±0.3	1.2±0.2	0.62±0.12
IgG_{2a}	4.6±0.8	3.8±1.0	0.77±0.10
IgG_{2c}	0.80±0.06	0.59±0.07	0.74±0.06
IgA	2.2±0.3	1.8±0.3	0.80±0.07
anti-SACHO	0.85±0.10	0.77±0.11	0.90±0.06
anti-DNP	0.77±0.11	0.41±0.06	0.53±0.08

[a]6-20 paired samples from hybrid or F344 rats were involved in each sera and ascites comparison. Randomly chosen samples from 3-7 weeks after the induction of ASF are represented.

Table 2. Cellular composition of F344 rat ascitic fluid

Cell type	Cells/ml ($x10^{-6}$)[a]		
	week 2[b]	week 3	week 4
Total leukocytes	8.4	7.1	6.2
Lymphocytes	0.9	0.8	0.4
Atypical lymphocytes	1.4	1.4	1.0
Polymorphonuclear leukocytes	5.8	4.9	4.0

[a]Mean number of cells from 13 animals are represented.

[b]Indicates week after induction of ascites

of paired samples obtained from individual animals over a four week time period. The similarity between ASF and serum again was evident in their closely paralleled regulation of anti-SACHO production (Figure 2). Although Id-1 is one of the predominate idiotypes naturally associated with anti-SACHO antibodies of rats, Id-1 and anti-SACHO production are regulated independently of each other (Olson and Leslie 1981). As is apparent in Figure 3, however, the same regulatory mechanisms affecting Id-1 production in serum also affected Id-1 production in ascites.

Cellular content of ascitic fluid. Cytocentrifuged preparations of ASF from F344 rats revealed several morphologically distinct cell types, the relative numbers of which varied with time and individual animals. The number of leukocytes in ASF decreased with time. Proportionately, there was a greater decrease in the number of typical lymphocytes than that of other cell types (Table 2). In addition, although very few eonsinophils were present in ascites, more were apparent in early ascitic fluid samples than in later ones.

Atypical lymphocytes represent a population of cells of which a significant percent age may be macrophages or monocytes. Also, among these atypical cells is a cell type difficult to classify morphologically for it is intermediary in structure between a macrophage and a typical lymphocytes. Nathan et al. (1977) isolated a similar nonphagocytic adherent cell from the peritoneal cavity of mice which was morphologically intermediate between small lymphocytes and macrophages. These cells appear to represent a subpopulation of B lymphocytes. The similarities between these cells in mice and those we have identified in

Figure 2. Comparison of anti-SACHO antibody concentrations in sera and ascitic fluids of 9 hybrid rats as determined by RIA analysis. Following the initial ip injection of 9 parts FIA to 1 part antigen (0.6 mg GASV in saline), additional injections of the same concentration of GASV in 9 parts FCA were given at weeks 2, 3, 4 and 5 (as indicated by arrows). At week 6, a 9:1 ratio of FCA to saline was injected ip. (Serum antibody ——— ; Ascitic fluid antibody -----).

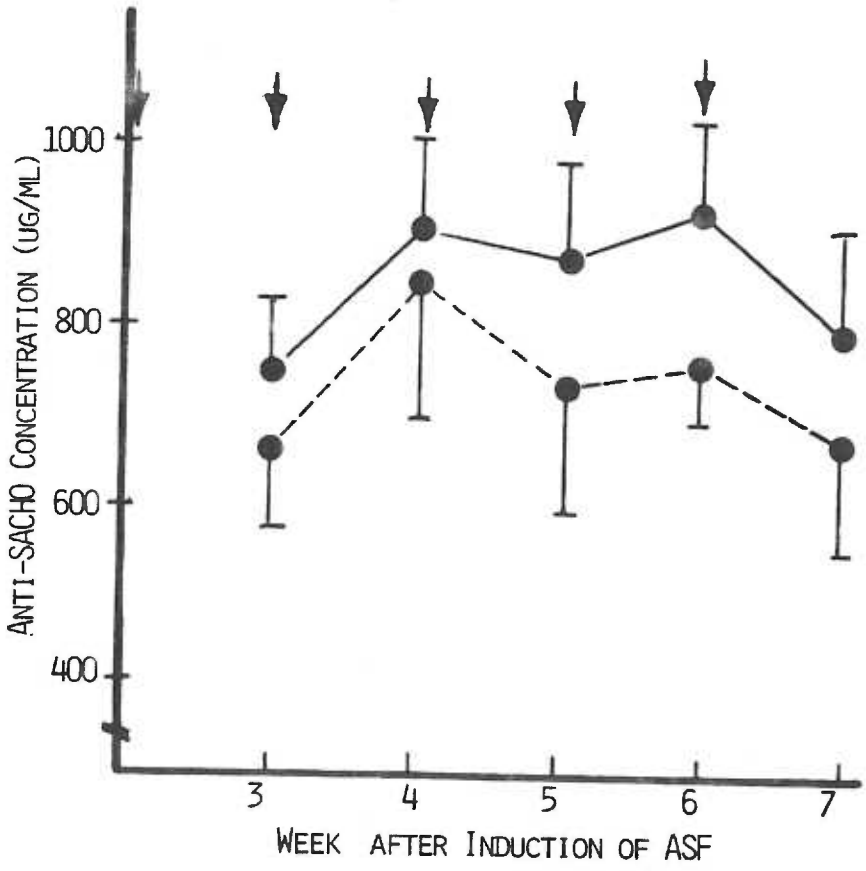
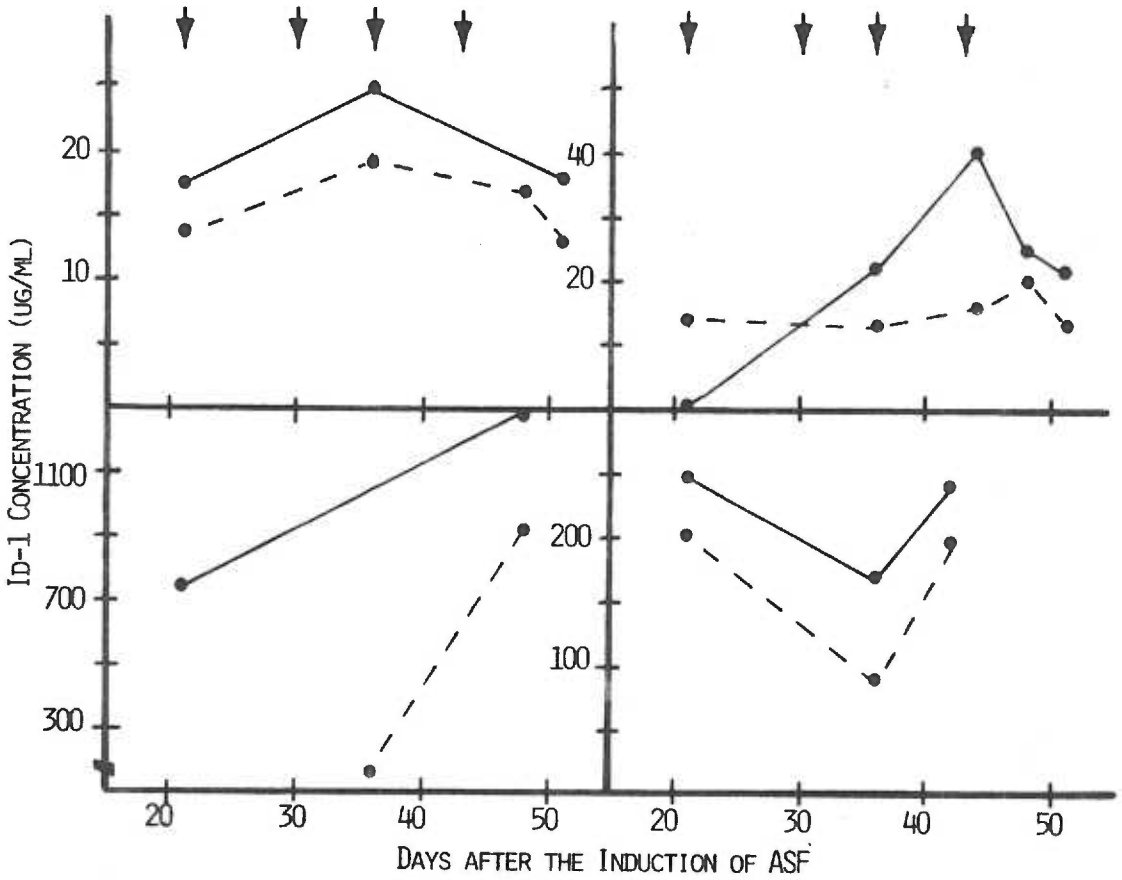

ANTI-SACHO CONCENTRATION (UG/ML)
1000
800
600
400
3
4
5
6
7
WEEK AFTER INDUCTION OF ASF

Figure 3. Comparison of Id-1 concentrations in sera (———) and ascites (----) of 4 hybrid rats relative to time. (Refer to Figure 2 for ascites induction protocol.) Additional injections of antigen in FCA from the 3-7 week period after ASF was initially induced are indicated by the arrows. Each graph represents 1 animal.


ID-1 CONCENTRATION (UG/ML)
20
10
1100
700
300
40
20
200
100
20
30
40
50
20
30
40
50
DAYS AFTER THE INDUCTION OF ASF

rats suggest that they may be closely related. Functional characteristics of this subpopulation in rat ASF have not been completed.

Factors affecting ascitic fluid volume

Sex influence on ASF production. In previous experiments we observed a difference between the volume of ASF produced by female and male rats. To substantiate this difference, we immunized 6 week old unprimed and adult GASV primed F344 rats with GASV/DNP. We used the DNP conjugated antigen in these studies to determine if ASF could also serve as an efficient source of anti-hapten (anti-DNP) antibodies. To assure maximal amounts of ASF were collected from each animal, rats were checked daily for ascites accumulation. In F344 rats, as well as all other strains tested, the average volume of ASF produced by females was greater than that produced by males (Figure 4 and Table 3). Over a 5 week period, adult female F344 rats produced an average of 65 ml of ascitic fluid (range: 48-80 ml), whereas F344 males produced an average of 29 ml (range: 18-46 ml). ASF collected from both sexes, however, did not contain significantly different concentrations of anti-DNP antibodies (females: 0.39±0.06 mg/ml vs. males 0.47±0.16 mg/ml). When ASF was induced in younger, 6 week old F344 rats, females again produced approximately twice the volume of ascitic fluid as male littermates. The onset of ascites accumulation was later in younger animals and the total volume collected was less than that obtained when ascites was induced in the adult F344 rats.

The effect of genetic variation on the production of ascites. There was considerable variation in the total volume of ascitic fluid

Figure 4. Comparison of volume of ASF collected from adult F344 female and male rats over a 5 week period. Standard error, and number of animals within each group are indicated. The enclosed panel represents the mean cumulative volume of ASF collected over the 5 week period.

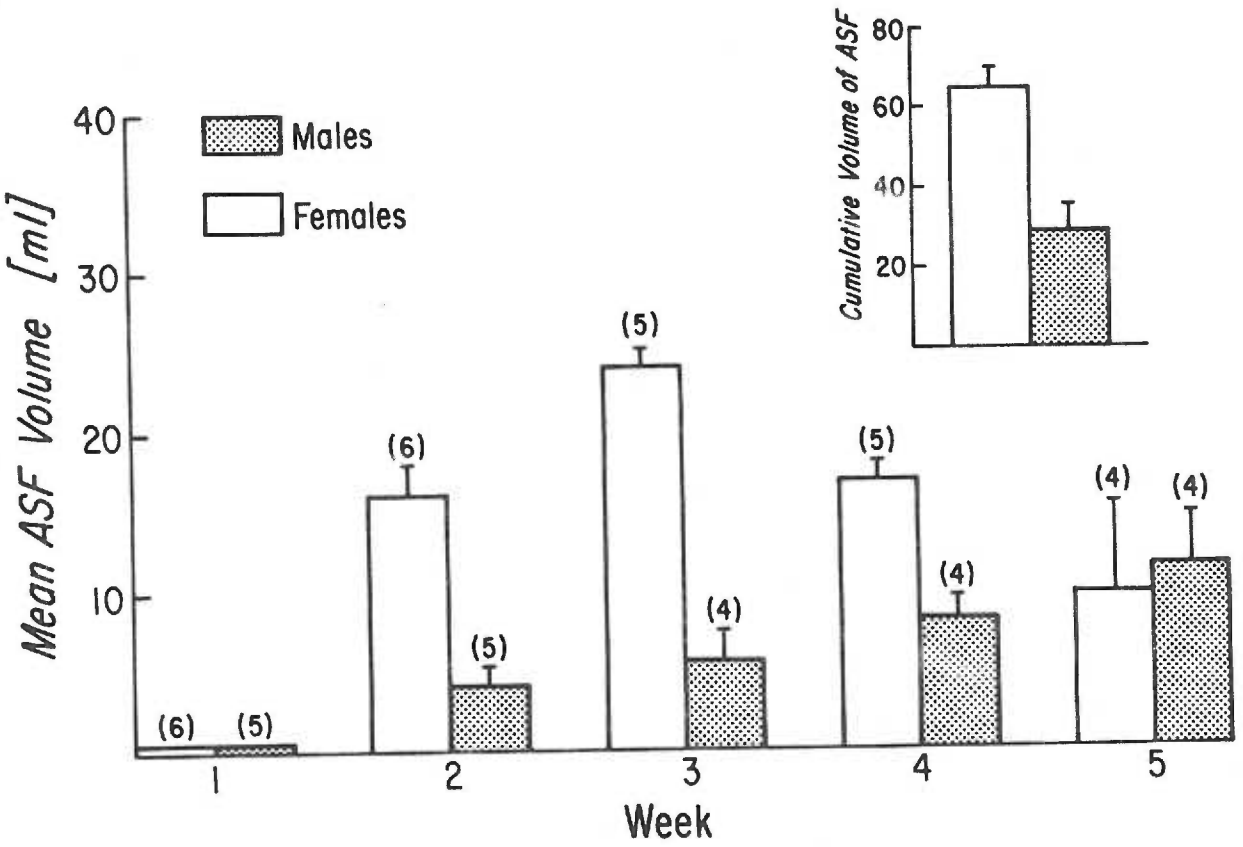

Mean ASF Volume [ml]
40
30
20
10
Males
Females
(6)
(5)
(6)
(5)
(5)
(4)
(5)
(4)
(4)
(4)
1
2
3
4
5
Week
Cumulative Volume of ASF
80
60
40
20

Table 3. Strain influence on rat ascitic fluid volume

Strain	Mean Cumulative Volume of ASF[a]	
	Females	Males
Copenhagen	52.2 (5)[b]	38.5 (2)
August	73.5 (4)	51.5 (4)
M520	37.0 (3)	9.0 (4)
F344 (Age - 6 wk)	44.7 (3)	24.2 (2)
(Age - 3 mo)	64.7 (6)	28.9 (5)
Hybrids (SD x Cop, SD x W/F)	126.5 (11)	48.9 (8)
(Aug x SD)	185.0 (2)	-

[a]Mean volume of ASF represents the mean of the maximum volume of ASF obtainable from individual animals during a 5-8 week period.

[b]Number of animals within each group.

obtained from the different strains of rats tested (Table 3). Of the inbred strains, August rats produced the largest volume of ascites. Hybrid rats, however (SD x Cop, SD x W/F, SD x Aug) produced considerably more ASF than any of the inbred rats. The initial onset of ASF production varied between strains (from 1 to 4 weeks after the initial injection of the antigen-adjuvant emulsion). Some animals never produced detectable amounts of ascitic fluid.

Discussion

It is becoming increasingly evident that immune responsiveness of an individual to a specific antigen depends not only on the inheritance of essential immune response genes, but also on the route and form of antigen administration (Vaz et al. 1977, Klinowska and Fargier 1978, Turcotte et al. 1978). Consistent with this is the observation that within an individual there appears to be unequal distribution or sequestration of certain subpopulations of immunoregulatory cells (Gearhart and Cebra 1979). As a result one can selectively induce a particular immune response if antigen is administered in such a way that it does or does not interact with specific immunoregulatory cells.

It was the purpose of these studies to determine whether mechanisms regulating immune responsiveness within ascites were similar or different than those regulating immune reactivity in the blood. When protein composition and specific antibody and idiotype concentrations of serum were compared to that of ascitic fluid obtained from the same animal, it was apparent that both ASF and serum were affected by the

same regulatory mechanisms. This is an important observation for it allows antibodies obtained from repeated ASF collections to be used to monitor immune regulatory mechanisms on-going within the circulation of individual rats. The potential of using ASF as a source of antibodies can be further realized from the collection of up to 3 grams of specific antibody from the ASF of a single rat at one time. This quantity of antibody enables one to monitor physical-chemical changes in specific antibody structure as it is being regulated in vivo by the immune system. We have successfully used ASF to monitor idiotype specific and anti-allotype (Dawson 1979) specific immune reactivity in rats.

In addition to monitoring humoral immunity, the rich cellular content of ASF permits the simultaneous analysis of cellular immune reactivity, without sacrifice of the animal. We have found 15% of the rat ascitic cells to be immunoglobulin positive (unpublished observation). Nisonoff et al. (1977), also, found mouse ascitic fluid to be a rich source of cells capable of transferring idiotype suppression to mildly irradiated A/J mice.

The quantity of ascitic fluid produced by individual rats varied with the strain and sex of the animal. Of the rats tested, SD x Cop, and SD x Aug and SD x W/F hybrid rats produced significantly more ASF than inbred strains, and female rats produced approximately twice the volume of ASF as males. When ASF was induced in younger rats, the onset of production was slower and the cumulative ASF volume was less than that of adults. In order to maximize the amount of fluid obtained from individual rats, animals should be tapped frequently. The

greatest volume of ASF was generally obtained early after induction, and the eventual limiting factor in ASF production was the accumulation of fibrotic tissue within the peritoneal cavity. We were, however, able to obtain significant quantities (50 to 130 ml) of ascitic fluid as late as 9 weeks after its induction, emphasizing its long-term duration. It should be noted that ASF induction or the accumulation of fibrotic tissue within the peritoneal cavity decreased the reproductive capacity of female rats. Such a procedure, would therefore not be recommended in animals destined for breeding.

By all criteria of analysis the mechanisms regulating protein composition of rat serum appear to co-regulate protein content of ascitic fluid. Similarities of proteins in ascites and sera of guinea pigs were also observed by Yaron et al. (1977). While maintaining these shared regulatory mechanisms, ASF induction has the potential to increase total antibody production of an individual rat by as much as 50 times. The increased antibody production by ASF induction most likely results from the transudation of intra- and extra-vascular proteins, and the generation or potentiation of antibody producing cells. Consistent with the transudation of vascular immune reactivity is the fact that ascites containing anti-SACHO antibodies can be induced by ip injections of saline in FCA to animals previously immunized intravenously with GASV. The large number of cells in ascites implies the stimulation of cell mitosis. Interestingly, a similar augmentation of an individual's immune potential can occur during pregnancy, where the fetus, rather than ascites, serves to absorb immune reactivity (Loke 1978, Scott 1976).

Both examples emphasize the effort the immune system is capable of exerting in order to maintain homeostatis.

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ABBREVIATIONS

Id-1: A public idiotype associated with rat anti-Group A streptococcal carbohydrate antibodies.

Anti-SACHO: Anti-Group A streptococcal carbohydrate antibodies.

GASV: Group A streptococcal vaccine.

MOPC 460: IgA, (κ) myeloma protein , specific for dinitrophenyl (DNP).

PC: phosphorylcholine

T15 or TEPC 15: Ig M (κ) myeloma protein which binds PC

S/D: Sprague Dawley outbred rat strain.

HPR: A strain of rats, originally of S/D origin, brother-sister inbred in our laboratory based upon the production of high levels of precipitating anti-SACHO antibodies.

LPR: A rat strain, also of S/D origin, brother-sister inbred in our laboratory based upon the production of low levels of precipitating anti-SACHO antibodies.

W/F: Wistar Furth inbred rat strain.

F344: Fischer 344 inbred rat strain.

iv: Intravenous

ip: Intraperitoneal

ASF: Ascitic fluid

NaSCN: Sodium thiocyanate

ppt: Precipitating

IA: Immunoadsorbent

IE: Immunoelectrophoresis

Abbreviations - Continued

ABC: Antigen binding capacity. The amount of radiolabeled Id-1^{+} IgG that binds to the anti-Id-1 preparation.

anti-GAT: anti-poly(Glu^{60}Ala^{30}Tyr^{10}) antibodies.

CGAT idiotype: Common idiotype associated with anti-GAT antibodies.

N-AcGln - N-Acetylglucosamine: The hapten recognized by anti-SACHO antibodies.

RIA: Radioimmunoassay

RRID: Reverse radialimmunodiffusion

SRBC: Sheep red blood cells

FCA: Freund's complete adjuvant

FIA: Freund's incomplete adjuvant

L-chain: Light chain

H-chain: Heavy chain

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