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HUANG, Jiin-Yuan, 1939-
QUANTITATIVE INHERITANCE OF IMMUNOLOGICAL RESPONSE IN SWINE.

University of Hawaii, Ph.D., 1977
Genetics

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QUANTITATIVE INHERITANCE OF IMMUNOLOGICAL RESPONSE IN SWINE

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN GENETICS

AUGUST 1977

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ACKNOWLEDGEMENT

I would like to acknowledge Dr. Robert C. T. Lee, Chairman, Joint Commission on Rural Reconstruction; Director F. K. Koh, Pig Research Institute, Taiwan; National Science Council of Republic of China; and Taiwan Sugar Corporation for their financial support, provision of experimental animals, and for the use of their facilities for this study.

ABSTRACT

Pigs were used as an animal model for studying the sources of variation in immunological response, under selected dose levels of immunization and at various stages during the development of immunity. Special emphasis was focused on the genetic component of the variation.

Bovine serum albumin was selected as an antigen to inject pigs at the age of 28 days. Based on the preliminary trials, two dose levels of BSA were chosen, namely 5 and 50 mg. The kinetics of immune response were studied to decide the time for taking blood samples for the main study. Three blood samples were taken at 14, 21 and 42 days after the first immunization, each representing the primary response, R_p, secondary response, R_s, and peak response, R_m, respectively.

A total of 802 pigs which were offspring of 18 purebred boars, each mated with six females, were used in the main study. Antigen binding capacity was used for measuring the antibody response. Data expressed as percent antigen bound was transformed by angle. No significant difference was found in the effect of sex, birth weight, weaning weight or pre-weaning daily gain on immune response. Pigs which received 5 mg of BSA gave consistently higher response than those receiving 50 mg of dose level in all three response measurements, i.e. R_p, R_s and R_m. The difference between the two dose levels and among litters in all responses were highly significant, but did not show any interaction between the dose level and litter. The intra-relationship of immune response measured at the three stages were found positively correlated.

The difference among sires was found to be statistically significant in immune response under the dose of 5 mg of BSA except for the primary response. Variations among dams within sires were significant only in the primary response. For pigs treated with 50 mg of BSA, no sire difference in the peak response was detected at the 1% significant level but the difference existed at the 5% significant level. Significant difference was found among dams within sires. The genetic contribution to the early response could not be important. As variation of immune response in 50 mg of BSA would be expected to manifest mainly environmental influences, this part of data was not used for estimation of genetic parameters.

The genetic variance of the three immune responses, 29, 59 and 51 percent of the phenotypic variance, showed an important effect on the secondary and peak response. The litter environment decreased in importance from 25% of the phenotypic variance in primary response to zero in the secondary and peak response. The individual environmental variance showed a stable variation among the three immune responses. The genetic correlations between the two adjacent stages of immune response, i.e. R_p and R_s, and R_s and R_m were considerably higher than that between R_p and R_m. Similar results were also found in the correlation measurements of individual environment and phenotypic measurement. The heritability obtained from sire and dam component combined for secondary and peak response were 0.51 ± 0.04 and 0.40 ± 0.04. The estimates were considered to be more precise than those calculated from other variance components. In conclusion, the multigenic control of immune response was demonstrated in the present study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

APPENDIX

CHAPTER 1

INTRODUCTION

Resistance to infectious disease in animals is dependent on complex responses of both a non-specific and specific nature. Non-specific resistance differs in species or races and strains of animals which may not provide optimal conditions to permit successful multiplication of pathogens and which may differ in their non-specific protective mechanisms. The differences of reticuloendothelial systems are also important in non-specific resistance. On the other hand, specific immunity is an adaptive response to an antigenic stimulus. It depends upon prior exposure to a foreign configuration and subsequent recognition of and reaction to it. There are two types of effector mechanisms which may occur in specific immune response: humoral immunity, which is the synthesis and release of free antibody into the blood and other body fluids, and cell mediated immunity, which produce sensitized lymphocytes. B-cells are the organization of lymphoid tissue into the bone marrow dependent compartment of cells responsible for antibody synthesis and the T-cells are processed by or are in some way dependent on the thymus. These two different small lymphocytes are not functionally independent. The T-cells appear to cooperate with B-cells in the generation of the humoral antibody response to certain but not to all antigens. These immunologic responses serve three major functions: defense, which involves resistance to infection by microorganisms; homeostasis, which is removal of damaged cell elements, and surveillance which involves perception and destruction of mutant cells.

It is realized that the immune response to infectious agents relate to resistance of disease. It has also been recognized that individuals afflicted with a disorder of antibody production are highly susceptible to infections. It is clear that immune response will be a predictor for disease resistance. And there are many evidences which indicate that the immune response is under genetic control. The differences in host resistance to particular pathogens often have a multigenic basis.

Studies of immune response in laboratory animals suggest that the variation in antibody response against the antigens is subject to genetic control and environmental influence. The genetic components of the variation are due to hereditary differences between individuals in the ability to recognize antigens and in the regulation of the amount of antibody produced. The environmental components may be due to the sum effects of physiological factors such as hormonal balance, stress, nutrition, and age, which influence antibody production. Although there are some evidences for monogenic control of specific response to simple antigens, the immune response genes being closely linked to histocompatibility genes, the response to specific antigens among different breeds or strains of animals has been shown to be under the control of multiple genes. It has also been demonstrated that selection for high and low response to specific antigens is possible. One remarkable finding is that the genetically selected high and low immune response lines maintain their immune response when injected with unrelated antigens. This separating effect of high and low level has also been shown in histocompatibility, in the level of different immunoglobulins, and in the rate of multiplication

and differentiation of antibody producing cells.

Since the immune response can be a predicator of disease resistance in animals, selective breeding for improvement of this particular trait can be expected, if this trait has a considerably high heritability. Though the heritability of immune response to certain antigens has been estimated under various conditions for several laboratory animal species, large animals such as pigs have never been used for such a study of quantitative genetic control of immune response.

The aim of the present study was to investigate sources of variation in immunological response, under selected dosage levels of immunization and at various stages during the development of immunity. Special emphasis was focused on the genetic component of the variation.

CHAPTER 2

LITERATURE REVIEW

2.1 Methods for detecting antibody response

Many sensitive techniques have been developed for detecting and measuring the interaction between antigen and antibody. Precipitin reaction is the interaction between specific antibodies and soluble antigens and has been used both in the quantitative determination of antibodies and for the identification and characterization of protein and polysaccharide antigens. Heidelberger and Kendall (1934) developed a method which was based on quantitative analyses of the protein nitrogen content of a series of antigen-antibody precipitates formed between a constant amount of antiserum and varying amounts of antigen.

Gel diffusion methods are simple and very sensitive for studying quantity or quality of antibody. The formation of an insoluble antigen-antibody complex becomes visible as a line of precipitation in the opalescent agar in the area where optimal concentrations for the formation of antigen-antibody precipitates are present. Single diffusion allows the antigen to diffuse, and the antibody is incorporated into the agar. This system can be performed in one or two dimensions. The single diffusion in one dimension is described by Oudin (1951). And the single diffusion in two dimensions has been adapted by Mancini, et al. (1965) for the quantitative determination of antigen concentration and is a very useful method for screening antigen concentrations of a large number of samples. Fahey, et al. (1965) have applied this method to determining the concentration

of different classes of immunoglobulins. When both antibody and antigen are allowed to diffuse toward each other, the system is called double diffusion which also can be performed in one or two dimensions. The double diffusion system in one dimension was originally described by Oakly and Fulthorpe (1953). The double diffusion in two dimensions allows comparison of different antigens with respect to their immunochemical identity, and cross reactivity. This method is also called the Ochterlony method (Ochterlony, 1948).

Immunoelectrophoresis developed by Grabar and William (1957), combines electrophoretic separation of an antigen mixture in agar with the precipitin reaction by double diffusion in two dimensions. This method is particularly useful for the examination of mixtures of protein antigens or mixtures of different classes of antibody.

Antigen-binding capacity method (ABC-method) developed by Farr (1958) is a method to measure quantitatively the amount of albumin antigen which binds specifically to an antiserum. The antigen-antibody complex is precipitated at 50 percent saturated ammonium sulfate, and this is the basis for measuring the antigen bound to the antibody or the antigen-binding capacity of the antiserum. The method is so sensitive that even a level as low as 0.005 μg antibody protein nitrogen can be detected.

Agglutinin reactions are used to detect antibodies directed against cellular antigens such as bacteria and erythrocytes which result in visible agglutination. Boyden (1951) has extended the use of this method to soluble antigens. This method called passive hemagglutination is based on the observation that erythrocytes

treated with dilute tannic acid absorb protein antigens on their surface and can be agglutinated by antibodies directed against the absorbed antigens (Boyden, 1951; Stavitsky, 1964).

Minden, et al. (1965) have compared some commonly used methods for detecting antibodies to bovine serum albumin in human serum. These methods are: the ammonium sulfate test (ABC-33), antigen-precipitating capacity (P-80), antigen-precipitating efficiency, passive hemagglutination, gel diffusion, passive cutaneous anaphylaxis (PCA), radio-immunoelectrophoresis and P-K passive transfer tests. Of the techniques used in this study, only radioimmunoelectrophoresis and the ammonium sulfate method detected the primary interaction between antigen and antibody. The other in vitro and in vivo tests depend upon measuring secondary events which, in varying degree, may occur sometime after the primary antigen-antibody reaction. In the detection and measurement of humoral antibody to BSA on the primary interaction between antigen and antibody, Minden, et al. (1969) also indicated the percentage of antigen bound to antibody as determined by 50% saturated Ammonium sulfate or by heterologous anti-r globulin was remarkably similar at various serum dilutions and at two antigen concentrations. Farr's method has been used for testing the kinetics of the reaction between antibody and bovine serum albumin (Talmage, 1960). This study indicated that ammonium sulfate is effective as a stopping agent in the reaction between I^{131} - BSA and antibody. Weigle (1961) has used this method in the study of the immunochemical properties of the cross-reactions between anti-BSA and heterologous albumins.

Green (1969) modified Farr's technique using high specific activity ^{3}H DNP-EACA to detect the low levels of anti-DNP antibody. The percentage of binding was calculated from the ratio: 1-(counts in unknown sera/counts in control samples) x 100. Chard (1970) described a radioimmunoassay procedure using ammonium sulfate precipitation for separating antibody-bound antigen from free oxytocin. A modification of the coprecipitation-inhibition technique (Farr's technique) in 50% saturated ammonium sulfate was used for detection of α_1-Fetoprotein (AFP) in the serum of rats (Oakes, et al., 1972). It was found to be sensitive and reproducible. A simple assay for quantifying IgE levels has been developed based on the observation that antibody-bound IgE is insoluble in 33% saturated $(NH_4)_2SO_4$, whereas unbound IgE is not. This method has been applied to human and rat IgE. Jones, et al. (1976) used Farr's technique to test for the measurement of antibody specific for Type III Pneumococcal polysaccharide (SSS-III). Linear regression analysis of the data was performed. For each point the x-coordinate was the dilution ($\log_{10}$) of the serum and the y-coordinate was the corresponding value for mean percentage of antigen bound. Using Farr's technique, a study of the genetic control of the antibody response to simple haptens in congenic strains of mice was carried out by Rathlum and Hildemann (1970). In this study the anti-TNP antibody level is expressed as "percent antigen bound" at fixed value of antigen added; this uses less serum than methods which compare the antigen bound relative to a fixed percent antigen bound.

Green and Benacerraf (1971) also used Farr's technique to assay the genetic control of immune responsiveness to limiting doses of protein and hapten protein conjugates in guinea pigs.

Although there are many methods for detection of primary interaction of antigen and antibody, the Farr technique has been used successfully in many studies of immune response. It has a number of advantages: 1) its simplicity allows rapid processing of a large number of samples, 2) the materials are relatively inexpensive, and 3) there are fewer problems related to variation between batches of reagents. Furthermore, the solubility of ammonium sulphate does not vary widely with temperature (Chard, et al., 1970).

2.2 Genetic control of immune response

Genetic control of immune response has been extensively investigated. In early experiments studies were made on genetic control with randomly bred animals to determine the responding or non-responding and the high or low responding individuals. Subsequently highly inbred animals immunized with complex antigens such as microorganisms or xenogeneic erythrocytes were used. More recently, immunization of highly inbred animals with chemically-defined antigens such as synthetic polypeptide molecules, have been studied for interpreting the genetic control of immune response (Mozes, 1972).

2.2.1 Multigenic control of immune response

As early as 1916, Cooke and Van Der Veer found that heredity could influence immune reaction in the development of some forms of human sensitization, such as hay fever, bronchial asthma, and gastro-enteritis which are genetically transmitted from parent to offspring as a dominant characteristic.

Based on tests involving the use of 5 inbred strains of mice, there has been evidence for definite genetic variation in the response

of mice to either egg albumin or pneumococcus polysaccharide used in the study by Fink (1953). This study also demonstrated different responses to two different chemically definable antigens, via different routes of injection, and in different ages of the animals.

Marked strain differences in the immune response to sheep red cells were found in mice. The hybrid and backcross experiments suggested that this was genetically controlled by at least three genes. (Playfair, 1968). Using synthetic polypeptide Glu^{56} - Lys^{38}-Tyr^{6} in 8 strains of rabbits and in New Zealand white rabbits (Gill, 1965), it was also found that there were significant differences in the amount of antibody produced in different strains.

James, et al. (1969) studied the primary humoral response of a number of inbred and random bred strains of rats. The results indicated that various hooded strain rats exhibited widely differing responses to bovine serum albumin. The conclusion of the study on genetic control of the immune response in rats to a synthetic antigen poly Glu^{52} Lys^{33} Tyr^{15} by Simonian, et al. (1968) was that the ability to produce antibody was transmitted as a complex, genetically controlled mechanism involving several genes.

Genetic studies on the ACI and F344 strains of rats, which were respectively the high and low responders to Glu^{52} Lys^{33} Tyr^{15} (Gill, 1970) and their F_1 and backcross offspring, showed segregation of the response and the genetic control with two independently segregated genes, one associated with antigenic recognition and the other controlling the quantity of antibody produced. In addition, there is some evidence of sex influence which may also be genetically controlled (Gill and Kunz, 1971).

Aggregation of the immunogen with a variety of agents enhanced the antibody response and delayed hypersensitivity reaction in the poorly responding F344 strain (Sloan and Gill, 1972). Similar antigenic complexes generated reduced antibody responses in the high responder ACI rats without affecting their delayed skin reactions.

Levine, et al. (1963) were the first to give evidence for the genetic control of the ability of guinea pigs to respond immunologically to synthetic antigens. It was found that 82% of the offspring from Hartley parents responded to DNP-PLL, and all of the offspring of non-responding parents were unable to respond to DNP-PLL. It was also found that strain 13 guinea pigs were non-responders and that strain 2 were responders.

McDevitt and Sela (1965) immunized CBA x C57F, F_1 x CBA and F_1 x C57 mice with (T, G)A-L. They found definite genetic control of the response that appeared to be the single major genetic factor with perhaps one or more modifying factors. They also found that the genetic control of the immune response to (H, G)-A--L appeared to be dominant and polygenic in the same way as was demonstrated for (T, G)-A--L (McDevitt and Sela, 1967).

After selective breeding of rabbits immunized with Group C-carbohydrate, complete segregation was achieved between a high-response population and a low-response population. Eichmann, et al. (1971) suggested that a limited number of genes control the degree of the immune response to the antigen.

Studies with the backcross inbred mice indicated multigenic control of the responsiveness against the GLA^5 and GLA^{10} (Maurer, et al., 1974). Immune response to GLO-GLT system in mice also indicated multigenic control (Merryman, et al., 1975).

In mice, the immune response to DNA was characterized as a dominant genetic trait and in backcross studies suggested that this immune response is under multigenic control (Fournie, et al., 1976). High-responder, SJL, and low-responder, DBA/1, mouse strains were given different doses of (T, G)-Pro-L in aqueous solution to investigate genetic control of immune response by using hemolytic plaque-forming cell (PFC) in the spleen as well as hemagglutination titers in the sera. There was no difference in the response and in the number of direct and indirect PFC in both the primary and secondary response between the strains when given an optimal dose (1 μg) of antigen. The direct and indirect PFC responses were lower in the low responder than in the high responder strain when given higher doses (10-100 μg) of antigen. After injection of a low dose (0.02 μg) of antigen, the low-responder strain produced more direct and indirect PFC than the high-responder strain. It was suggested this I_r-gene regulated the balance between stimulation and suppression according to the dose of antigen given (Jormalainen, et al., 1975).

Using parents, F_1 hybrids and backcross offspring of inbred mice, a study was carried out to measure the amount and relative affinity of antibody produced in response to protein antigens injected in saline. The results of this study suggest that the antibody affinity is under polygenic control. They also indicate that strain-related variation in the amount of antibody is independent of the relative affinity, and that these two parameters are under independent genetic control systems (Steward and Petty, 1976).

In certain conditions of immunization, C57131/6 mice are high responders whereas AKR mice are low responders to bovine gamma globulin

(BGG). When mice were given BGG alone, a dose higher than 0.1 mg of BGG reduced the difference between the high and low responders. When this dose (0.1 mg) of BGG was given with adjuvant, AKR mice showed an increase in the response, but the C57131/6 did not. When mice were given a low dose of BGG (1 μg) with adjuvant $Al(OH)_3$, C57131/6 showed high antibody titer, but AKR mice showed very low titer. The results suggest that the enhancing effect of adjuvants on the immune response to BGG is dependent on the dose of antigen, and indicate a quantitative genetic control of the immune response to BGG (Gherman and Sulica, 1976).

2.2.2 Selection for antibody response

The selective breeding for high and low immune responder lines of mice to multideterminant strong immunogens such as heterologus erythrocytes has shown that multiple genes are involved in controlling this particular trait (Biozzi, et al., 1970). After 20 generations, the mean aggultinin titers against sheep erythrocytes showed a marked difference in the two lines (Biozzi, et al., 1971).

The most remarkable finding in the selective breeding was that high and low lines were shown to be separated with respect to their antibody responses to antigens unrelated to those used in the selection process, such as the O and H antigens of salmonella typhi (Biozzi, 1970; Prouvost-Danon, et al., 1971), Limulus polyphemus hemocyanin, and bovine serum albumin (Biozzi, 1971). The two lines of mice also differ in their ability to synthesize humoral antibody against histocompatibility antigens (Biozzi, 1971). In the induced tumor cytotoxic study, Biozzi (1971) found that the high line of mice gained high titer of cytotoxic antibody against "low" spleen cells, while the low line gained a low

titer of weakly cytotoxic antibody. When injecting mice with benzopyrene, more tumors were found in the "low" line as well as a faster rate of tumor growth.

A marked interline difference in the serum concentration of all classes of immunoglobulins has been demonstrated after antigen stimulation. The levels are uniformly lower in the low responder than in the high responder mice (Biozzi, et al., 1970). Guercio and Zola (1972) also found that the two lines of mice showed a great difference in their ability to respond to primary and secondary immunization with dinitrophenyl groups coupled to bovine r-globulin.

All these findings suggest that selection operated primarily at the level of genes involved in antibody synthesis, irrespective of immunological specificity (Biozzi, et al., 1972).

Selective breeding for agglutinin production to heterologous erythrocytes, sheep erythrocytes (SE) and pigeon erythrocytes (PE), was carried out on a randomly bred population of albino mice. In order to eliminate the maternal antibody interference, two types of selection were performed in this study. The results of both selections indicated polygenic regulation of the aggultinin synthesis. The heritability of this trait was estimated to be between 0.18 and 0.36 (Feingold, et al., 1976).

Katz and Steward (1975) selected randomly bred TO mice into high and low lines based on the relative affinity (K_R) of antibody produced in response to protein antigens. After four generations of selective breeding, the K_R on the two lines showed a highly significant difference. The results indicate that antibody affinity is genetically controlled and is independent of the antibody level.

2.3 Heritability of immune response

It has been found that there is a variability in the immune response among animals. This variability suggests that hereditary difference may contribute to the antibody response. In general a quantitative trait shows genetic and environmental effects. Sang and Sobey (1954) have studied the inheritance of the extent of antibody response to tobacco mosaic virus (T.M.V.) and bovine plasma albumin (B.P.A.) in rabbits. Antisera were collected 10 and 13 days after the second injection of B.P.A. and were titrated, using the optimal proportion method. T.M.V. was injected immediately following the B.P.A. injection. Anti-T.M.V. was titrated by the equivalence zone method. Using the regression of mean immune response of unselected offspring to the mean immune response of their parents, the heritabilities of secondary response to T.M.V. and B.P.A. were 0.876 and 0.09 respectively. From the frequency distribution of response to B.P.A. in the two different populations, it was concluded that the populations differed in their ability to produce antibodies. The correlation between responses to T.M.V. and B.P.A. is 0.27 ($p < 0.01$). With diphtheria toxoid there is a definite and significant correlation between primary and secondary responses. There is no correlation between primary and secondary response to tetanus. In the study described above, Sang and Sobey point out certain necessary conditions for further study: a) the antigen used must be pure, b) the injection regimen must be standardized and simple, c) both primary and secondary response curves must be determined, and d) the titration method must be done with a low inherent error.

Sobey (1954) used equivalence zone method to titer the anti-T.M.V. antibody in rabbits. Measured by a midparent offspring regression, the heritability of secondary response to T.M.V. is 0.876. The response to T.M.V. was measured by the "equivalence zone" technique, while the B.P.S. was measured by the "optimal proportions" method. The measurements were made in each case with samples of the same serum from the same bleeding, thus, the environmental variation would be the same. Why did heritability of response vary significantly from one experiment to another? Sobey and Adams (1955) conducted another experiment in an attempt to answer the question. They used sheep red cell, which is a complex antigen, and measured the response using haemolytic titration. One hundred twenty pairs of albino mice were used in this study. The time response curves were determined before they chose day 3 and 6 as the best time for bleeding. They found that there was a significant difference in sex. The responses were corrected for constant age and sex, and regression of offspring on midparent was calculated using corrected figures. The heritability of antibody response to S.R.C. was found to be 0.28 at 3 days and 0.11 at 6 days. The correlation between these two periods was found to be $r = 0.57$. Their discussion indicated that the method of measuring response might be inadequate and, in the conclusion, suggested that the detailed knowledge of the complexity of the antigens and the measuring method must be of concern in such a study.

Due to a large sampling error in Sobey's paper (1955), Claringbold and Sobey (1957) studied the inheritance of antibody response with a different approach in order to estimate the heritability using the experimental design of daughter-dam pair in albino mice. In the

study of primary response, a 4 x 2^3 factorial experiment was used to calculate independent effect of line, dose, routine of injection, weight, and time of testing on response. The experimental design for secondary response is a 4 x 2^5 factorial experiment. The analysis of variance showed that the responses are very strongly dependent on dose, and that the dose response slope is a function of age. The heritability of primary response is 0.428, while the secondary response is -0.074. The correlation between the primary response of dams and secondary response of daughter, or vice versa, is not significant. The variability of primary response is about twice that of secondary responses. Another experiment was carried out by Sobey and Adams (1961) to determine the heritability of antibody responses using V_i and O antigens of Rhizobium meliloti and MEL and LEE Strains of influenza virus. The antihaemagglutination and neutralization methods were used in this study. Heritabilities were obtained by doubling the correlation of daughter with dam scores. A heritability of -0.012 for V_i and 0.78 for O antigens was obtained. When testing for response of V_i with both the V_i and O antigen present, the heritability is low; but when the V_i antigen is destroyed by heating, the only measurable response is that to the O antigen and here the heritability of response is high. The heritability for MEL virus was 0.60 by anti-haemagglutinin and 0.16 by the neutralizing potency test and may indicate that the tests did not measure a single antibody but a complex of antibodies. It suggests that such study requires using purified antigens which will elicit a single antibody response.

The heritability of antibody response to sheep red cells in mice, which were selected for high and low responders for 21 generations,

was estimated and was found to be 0.36 (Biozzi, et al., 1970).

2.4 Environmental factors in immune response

The variation in the immune response against the antigens is subject to genetic control and environmental influence. This review is concerned with dose, frequency and route of administration of the antigen, hormonal balance, age, stress and nutrition.

2.4.1 Dose, frequency and route of administration of antigen

Immune response is dependent on the dose of antigen, but optimum dose is required for the best response. The effect of antigen dose and time after immunization on antibody affinity was studied in a haptenic system by Eisen and Siskind (1964). It was found that affinity increased progressively with time after immunization, and that this increase was greater with lower doses of antigen. High dose of antigen resulted in early immunization in higher concentrations of antibody, followed later in the immune response by decreased serum levels of antibody as compared with lower doses of antigen. This effect of high doses of antigen can be attributed to induction of immunological tolerance (Siskind, 1968).

Low zone tolerance can probably be obtained under favorable circumstances with all low and medium molecular weight soluble proteins such as serum protein antigen (Mitchison, 1968). Mature mice were injected repeatedly with various doses of bovine serum albumin (BSA) and then measured for antibody response. Mitchison (1968) found that mice given repeated low doses of BSA became tolerant; mice on medium doses

of BSA became sensitized and gave a good antibody titer on challenge while those on high BSA doses were tolerant. Thus, there is a "low zone" and a "high zone" for tolerance.

In addition, these high doses of antigen affect B as well as T lymphocytes instead of only T lymphocytes as in low zone tolerance (Mitchison, 1970).

A study of the response of guinea pigs to bacteriophage ϕ x 174 (Uhr, 1962) noted that the secondary response evoked about one hundred times as much antibody as the primary response and reached a peak earlier. The primary response is composed mainly of 19s antibody, and the secondary response is composed mainly of 7S antibody (Uhr, 1967). Nossal, et al. (1965) have emphasized the similarity between the two responses. If the dose of antigen is adjusted, primary and secondary responses can be made to resemble each other quantitatively. It has also been shown that 19S antibody is produced first, followed by 7S antibody (Wei and Stavitsky, 1967).

Antibody produced in the secondary response tends to have a higher affinity than that produced in the primary (Steiner and Eisen, 1967).

Many routes of immunization have been used to produce antibody. Some routes favor induction of delayed hypersensitivity, some favor preferential synthesis of various immunoglobulin classes, and some favor induction of immunological tolerance. The site at which the immunogen is administered should influence the antibody production. Normally, subcutaneous, intra-muscular injections are used (Hurnand Landon, 1970).

2.4.2 Sex hormone and age in immune response

The hormone balance influences the maturation of the immune system as well as general immunological reactivity during periods of stress. Somatotrophin, thyrotrophin, corticotrophin and gonadotropin and the endocrine secretions have been reported to control the immunological development and have been reviewed elsewhere (Batchelor, 1971; White and Goldstein, 1972; Hudson, 1974). Estrogen increases the phagocytic activity of the reticuloendothelial system, but androgens and progesterone do not (Nicol and Bilbey, 1960). The estrogens also increase the effect on lymphoid tissue structure and functions, but the androgens do not. The estrogens have been observed to enhance antibody production and levels of serum immunoglobulin in females in comparison with levels in control groups of males (Butterworth, 1967; Terres, 1968). It was shown that changes in sex hormone levels exerted a marked influence on immune responsiveness and stem cell differentiation (Eindinger, 1972).

The effects of age on the immune response were studied in mice. A decline in the antibody forming activity was seen after 20 weeks of age (Makinodan, 1972). The immunologic inadequacies of the aged reflect both intrinsic deficiencies in the immunocompetent cell population and extrinsic deficiencies in the environment of cells.

Studies on humoral immunity in aging showed that 90% of the decline in the humoral response is intrinsic to the immunocompetent cells. Although the stem cells as well as the accessory cells do not show deficiency with age, the functional capacity of both T and B lymphocytes is markedly depressed (Nordin and Makinodan, 1974). It

was found that BALB/C and C3H mice could mount high level plaque forming cell (PFC) response to SIII at various age through 110 weeks, whereas, the level of the SJL/J PFC responses began to decline by the age of 42 weeks through the age of 80 weeks. Also the suppression T cell activity was apparently greater in young BALB/C mice than in older BALB/C mice (Smith, 1976).

The effects of age on the immune response were studied in highly responding and poorly responding strains of inbred rats. Changes in the immune response associated with age were found only in the highly responding strain. The antibody response fluctuated up to 10-12 weeks of age, showed no significant change up to 60 weeks of age, and decreased thereafter. The factors influencing antibody formation at different ages appear to modulate the genetically set capability of the immune system (Kunz, 1974).

2.4.3 Stress and nutrition in immune response

Stress appears to have an important influence on disease resistance. Much work on stress and susceptibility to disease has been conducted in man and in laboratory animals. Detectable changes in immunological mechanisms include impaired antibody responses (Solomon, 1969b). Little is known about the mechanism of this association.

Protein-calorie malnutrition has been a widely known cause of impaired immunoglobulin synthesis. Experimental animals deprived of proteins showed an impaired ability to respond to primary immunization (Kenney, et al., 1970). A study showed that protein supplementation of diets led to a rapid recovery of antibody-producing capacity

(Mathews, 1972). The reticuloendothelial system with phagocytic function is an important accessory to antibody formation. This system is often impaired in protein-calorie malnutrition (Arbeter, 1971), The clinical and experimental evidence has suggested that increased incidence of disease in protein deficient subjects is not always explained by a reduction in antibody synthesis. Many factors may contribute to impairment of the resistance. The cell-mediated immune response is probably most important (Smythe, 1971; McFarlane, 1971). Vitamin A deficiency in pigs lead to a reduced response to Salmonella Pullorum antigen (Harmon, 1963). Vitamin A also enhanced the response of mice to sheep erythrocytes and led to a more rapid rejection of skin grafts (Jurin, and Tannick, 1972). Deficiencies of vitamins of the B group have been associated with a reduction in antibody production to antigenic stimuli (Axelrod, 1971). Vitamin C has little effect on antibody production (Kumar and Axelrod, 1969).

Physical environment such as climate and weather appear to have most profound influences on immunological status and disease resistance. Many infectious or immunological disorders of man and domestic animals show seasonal cycles presumably associated with the thermal environment (Top, 1964). The faster catabolism of antigen and earlier appearance and higher levels of circulating antibodies were found in the cold-exposed group (Rose and Sabiston, 1971). The suppressive effects of many pathogenic organisms on the general immune response of the host have been demonstrated. Parasitic infectious have shown immunosuppression (Hudson, 1973b). The role of immune suppression induced in tumors by viruses has been reviewed by Dent (1972). In

addition to viruses, mycoplasma (Roberts, 1972) exhibits immunosuppressive characteristics.

2.4.4 Antigenic competition in immune response

The depression of immune response to one antigen may occur as a result of the injection of a second antigen. In mice injected intravenously with antigen in incomplete Freund's adjuvant, there was competition between BGG and ferritin (Adler, 1964). It was shown that large doses of BGG depressed antibody production in response to ferritin, although smaller doses actually increased the level of ferritin antibodies. Competition could still be demonstrated if the BGG was given before the ferritin. The bovine serum albumin and haemocyanin in a comparable type of experiment also gave the same conclusions (Cremer, 1963). There was no diminution in the antibody production to sheep red blood cells (SRBC) when horse red blood cells (HRBC) were given simultaneously. Prior injection of HRBC decreased the response to SRBC (Radovich and Talmage, 1967). The mechanism of antigenic competition is still not conclusive.

2.5 Studies on immune response in pig

2.5.1 Transmission of passive immunity from mother to offspring

Due to the structure of the placenta of the pig, there is no maternal r-globulin present in the blood of newborn pigs before they suckle colostrum (Kim, 1964; Myers and Segre, 1963). Colostrum and milk are the only sources of maternal immunity for newborn pigs. The pigs utilize the immunoglobulin from the colostrum during the first 24-36 hours after birth. After this period, the intestinal absorption

will cease (Miller, 1962; Speer, 1959). The pig colostrum contains all three immunoglobulin classes; IgG, IgM and IgA. Although the antibody in colostrum is mainly IgG and is three times as concentrated as in the serum, it decreases rapidly during the first week of lactation. IgA is four times as concentrated in the serum and declines during the first 24 hours of lactation, but not as markedly as IgG or IgM. It becomes the predominant immunoglobulin in milk (Bourne, 1976; Porter and Noakes, 1969).

This passive immunity is gradually replaced by active immunity in the young pigs. By using sodium sulphate precipitation and free boundary electrophoresis, Jakobser and Moustgaard (1950) and Rook (1951) observed the euglobulin content in pig serum from birth to maturity. They found that the euglobulin content rose to a maximum after suckling and then fell to a minimum between 20 to 30 days of age, thereafter, rising gradually until adulthood was reached, indicating that the r-globulin level falls to a minimum at 3 weeks of age. Nordbring, et al. (1957) found that r-globulin in the serum decreased to a minimum at about 4 weeks of age and thereafter increased gradually. From these results, it is indicated that the time of minimum concentration of r-globulin in pig serum is between 3 and 4 weeks of age.

2.5.2 Active antibody production

Brown (1961) pointed out that the serum antibody of baby pigs which absorbed the Serratia marcesceus from high titer colostrum can be detected through 6 weeks of age. This differ from the findings of Miller (1962) who discovered that absorbed antibodies are retained in measurable concentration for about seven days.

Curtis and Bourne (1973) studied the time of onset of immunoglobulin in pigs. They concluded that there was no IgG produced during the first 14 days and no IgA until the first week of age. Immunoglobulin-containing cells in the intestinal tract have been found on the tenth day of age (Allen and Porter, 1973), and all classes of immunoglobulin also have been found in the duodeum during the first week of life (Brown and Bourne, 1976). Miller (1962) found that the capability to produce the serum antibodies to Salmonella Pullorum and serum immunoglobulins increase until pigs were 3 weeks of age as well as a 20 fold increase in serum antibody titer in pigs which were 6 weeks old. Hoerlein (1957) used killed Brucella abortus, or sheep red cells, or bovine serum or egg albumin to innoculate the young pigs at the age of 3 to 8 weeks and administered a booster dose at 3 weeks after the first injection. The pigs were either allowed to suckle, deprived colostrum, or received immune colostrum. Pigs under 8 weeks of age, which were deprived of colostrum, did not produce antibody in measurable quantity, except to sheep red cells, whereas those that had received colostrum (non-immune) did produce antibody at 3 weeks old and this increased markedly up to 6 weeks. Antibody production of pig at ages 3 and 6 weeks interfered with immune-colostrum.

Three weeks-old colostrum-deprived pigs failed to produce antibody to diphtheria toxoid and responded only slightly to tetanus toxoid. Those fed with colostrum responded strongly and within a week to both antigens. The colostrum deprived pigs showed the intermediate response when injected with combined toxoids with a minimum amount of pig serum from pigs hyper-immune to both antigens. Removal of the

antitoxins from the hyper-immune serum resulted in loss of its stimulating effect. It was also found that the serum from 15 weeks old pigs provides a greater stimulus for active antibody production in a colostrum deprived pig than the serum from 9 weeks old pigs. Segre and Kaeberle (1962a, 1962b) found colostrum-fed pigs produced diphtheria antibodies after intraperitoneal innoculation of the toxoid at birth, but the production of antibody to tetanus toxoid was depressed. Colostrum deprived pigs produced tetanus but not diphtheria antibodies. These results indicated that a degree of transplacental transfer of maternal antibody of tetanus, but not of diphtheria, are present in the sow's blood. Kaeberle (1968) found that colostrum fed pigs did not produce antibody in response to tetanus, but responded better to antigens than colostrum-deprived pigs, and the response was enhanced by the presence of a small amount of specific antibody. It was concluded that passive immunity from colostrum may inhibit the development of active immunity for the first 3 weeks of life, but its presence at a low level, thereafter, enhances the production of antibody.

Kim, et al. (1966) found that no immunoglobulin could be detected in sera of germfree colostrum-deprived miniature piglets at birth. A single antigen showed a greater primary immune response than a multiple antigen. This indicated antigenic competition. The antibody response to a single injection of the phage in 1 month old piglets was identical to that of the adult sow. The first antibody produced in response to the specific antigenic stimulus was 19S (r_1)-immunoglobulin which developed later in the piglets, and the 19S immunoglobulin was distinct from r M-macroglobulin (Kim, et al., 1966).

Although pigs can produce antibody during the first week to antigenic stimulus at birth, a more normal time for active immunization is at 3 weeks old, and the capacity of increasing the immune response is not until at least 6 weeks old (Brambell, 1970).

It was concluded that colostrum and traces of specific antibody in passive transmission may play an important role in active immunity, but an amount of passive immunity to a specific antigen greater than a trace amount would interfere with active immunization as long as it persists.

2.5.3 Sources of variation in immune response in pigs

Perry and Watson (1967a) studied sources of variation in the uptake of a marker antibody by piglets from 26 litters sired by 10 boars. Analysis of variance in efficiency of antibody absorption showed that 25% of total variance was due to time of dosage, 13% to dosage concentration and 6% to sire. Half of the total variance was within subclass. Further analysis showed that three-fourths of this variance within subclass was due to between litters and only one fourth was due to within litter. This indicated that the maternal effect was a very important source of variation. Sixteen sows at parturation showed marked differences in serum antibody to Salmonella pullorum antigen which was used to immunize the sow in late pregnancy. The piglet serum antibody titers measured at twelve hours after birth showed a positive relation to the serum titers of their mother. No relationship was found between colostral titers and the titers in sow or piglet sera. The titers of the colostral samples showed marked variation both between different teats and different sows. The piglet

and litter weight gain from birth to seven days of age were negatively related to sow and piglet serum titers. Those piglets with high serum antibody titers at 12 hours after birth showed faster growth rate and lower mortality than those with low serum titers (Perry and Watson, 1967b).

The breed differences of pig in the immune response to sheep red blood cell were studied by Buschmann, et al. (1974). The immune response to SRBC as determined by the number of plaque forming cells (PFC) in the spleen of pigs revealed a significant difference among breeds. A significant seasonal influence on the number of PFC was observed in immunized animals. The breed differences in immune response to SRBC were supposed to be due to different genetic background.

Using the phage neutralization technique to detect the anti-hapten antibodies in the pig serum from 8 different breeds after immunization with DNP-BSA, it was found that 49.0% of pigs' serum showed no antibody activity, 38.0% showed weak antibody titer and 13.9% showed high antibody titer. The frequency distribution of response showed breed-specific differences (Buschmann, et al., 1975).

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental animals

Pigs were used for experimentation in the present study. All animals, raised in complete confinment, were randomly selected from a herd of some 3,000 breeding females maintained at the Animal Industry Research Institute, Taiwan Sugar Corporation (TSC). At birth, the pigs were allowed to suckle colostrum from their mothers and were given creep feed from the first week until weaning at 28 days of age. After weaning, all the litters were moved to another facility. Management and feeding were uniform through all the experiments. In this herd, hog cholera vaccine was administered routinely to immunize the pigs at the age of 49 days.

3.2 Immunization

Bovine serum albumin (BSA) was selected as an antigen, since it is a soluble, pure protein and is well known as a good immunogen when used with Freund adjuvant. This protein antigen, obtained as a commercial product of the Sigma Company, was emulsified in an equal volume of complete Freund's adjuvant (CFA) (Difico Laboratories) for the first injection and the booster dose was made with BSA in incomplete Freund's adjuvant (ICFA). Immunization was made by deep intramuscular injection on the inside of the pig's thighs near the lymoph node.

3.3 Collection and preparation of blood sample

A schedule of drawing blood was established in each experimental design. Ten ml of blood were obtained from the anterior vena cava and

allowed to clot overnight at a temperature of 4°C in the refrigerator. The blood was subsequently centrifuged at 1,500 rpm, 15 minutes in 4°C. The sera obtained from the supernatant were inactivated at 56°C, for 30 minutes and transferred into two vials stored at -20°C for antibody assay.

3.4 Preliminary trial

3.4.1 Trial 1: Dose level of antigen

Three dose levels were used, namely, 0.5, 5.0 and 50 mg/ml of BSA. A total of fifteen 28-day old pigs at weaning from three litters were randomly assigned to the respective levels. A booster dose was given two weeks later. Blood samples were collected from each animal every week after first immunization for a period of five weeks. Each sample was examined for antibody response to BSA by using gel diffusion and passive hemagglutination.

3.4.2 Trial 2: Kinetics of anti-BSA response in pigs

Five 28-day old pigs were used. Fifty mg/ml of BSA was given in the first injection with a booster dose two weeks later. Blood was drawn from each pig every three days for two months and the antisera were analyzed for immune response by using the antigen binding capacity method, details of which were given in 3-6. Non-linear regression analysis of percent antigen bound on the days that response was measured was used to plot the response curve.

3.5 Main experiment design

In the main experiment three treatment combinations were used.

Each litter selected for study was divided into two groups of approximately equal number with similar sex composition, and was randomly assigned to one of the three treatments. For the first treatment half of the litter received no antigen and the other half 5 mg/ml of BSA. The second combination specified no antigen and 50 mg/ml of BSA for the two groups of each litter assigned. Two dose levels of BSA, namely, 5 and 50 mg/ml characterized the last combination. A total of 802 pigs which were offspring of 18 purebred boars each mated with six crossbred females were used. Table 3-1 gives the mating scheme in which litters were assigned to various treatment combinations of BSA dosage. The use of no antigen was specifically designed to study the effects of subsequent BSA immunological response to the hog cholera vaccine which was scheduled for every animal at seven weeks of age. Figure 3-1 illustrates the plan of immunization and blood collection. The first dose of BSA was injected at the age of 28 days and a booster dose was given two weeks later. The blood samples were collected at 14, 21 and 42 days in order to study the immune response in different stages of immunity development.

3.6 Measurement of immune response

3.6.1 Gel diffusion

This method was employed in Trial 1. One percent agarose was poured on glass plates and allowed to solidify at room temperature. Opposite wells were cut into the agarose by suction. The central well was filled with solutions containing anti-BSA serum and the peripheral wells with BSA solution. During the next day, a precipitin line was expected to be visible between the wells if there was

Table 3-1. Experimental Design

Sire		S1						S2								S18
Dam		D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D108
BSA Dose	0	x	x	-	x	x	-	x	x	-	x	x	-	x	x	
(mg/ml)	5	x	-	x	x	-	x	x	-	x	x	-	x	x	-	x
	50	-	x	x	-	x	x	-	x	x	-	x	x	-	x	x

S = sire

D = dam

x = inject

- = not inject

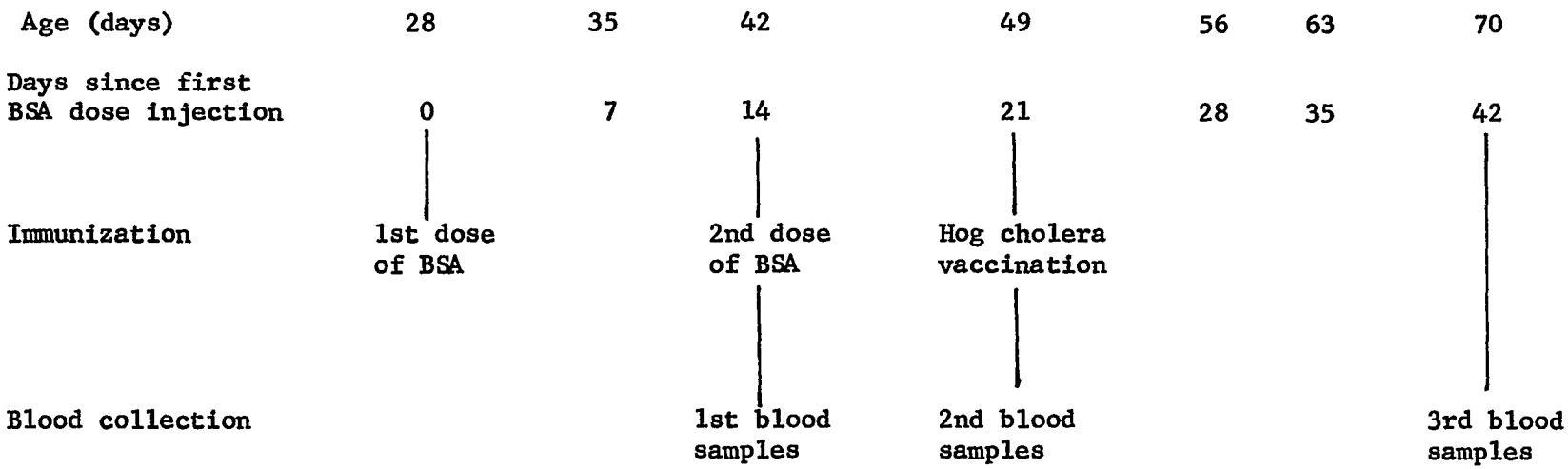


Figure 3-1. Schedule of Immunization and Blood Collection

anti-BSA antibody in the test antiserum.

3.6.2 Passive hemagglutination

The method of passive hemagglutination (Boydin, 1951) was also used in Trial 1, based on the observation that the tannic acid treated erythrocytes which would absorb protein antigen on their surface and the antibody could agglutinate with this sensitized cell directed against the absorbed antigen.

Sensitized cells were prepared according to Boydin (1951) and Campbell (1963). Sheep red blood cells (SRBC) were first washed three times with phosphate buffered saline (PBS) pH 7.2. Ten ml of freshly prepared solution of 1:20,000 dilution of tannic acid in PBS, pH 7.2 and added with equal volume of SRBC in PBS pH 7.2. After incubation for 15 minutes at 37°C, the SRBC were washed with PBS and suspended in equal volume in a solution containing 1 mg BSA per ml PBS, pH 6.4. Following incubation with antigen at 37°C for 15 minutes, the SRBC were centrifuged and washed with PBS, pH 6.4, to remove nonabsorbed antigen and finally suspended in 1% normal rabbit serum.

The absorption of nonspecific antibody was made by taking 0.4 ml antiserum mixed well with 0.02 ml washed pack SRBC in a small tube. After incubation at 4°C overnight, the cells were centrifuged and the supernatant was transferred into another tube kept in 4°C for the test.

Twofold serial dilutions of each antiserum using microtiter were prepared in the plate wells using 1% NRS as dilutent. An equal volume of tanned sensitized SRBC was added to each well. The plates were

read after incubated at 37°C for 1 hour and kept at 4°C for reading the following morning. The titers were expressed as the highest dilution which gave a positive agglutination.

3.6.3 Antigen binding capacity (ABC)

The Farr method (Farr, 1958) was selected for the measurement of antibody level in the blood. This method was originally developed to measure quantitatively the amount of albumin antigen which bound specifically to the globulin fraction of an antiserum. It determined indirectly both precipitating and nonprecipitating antibody activity, based on the differential solubility of albumin and globulin in 50% saturated ammonium sulfate (SAS). It was observed that soluble antigen-antibody complexes were precipitated at a 50% SAS concentration, whereas, albumin antigen which is not complex to antibodies remained in the supernatant. When a constant amount of a labeled antigen I^{131}-BSA was added to serial dilutions of anti-BSA, until the I^{131}-BSA excess was achieved, spontaneous precipitation of I^{131}-BSA and antibody aggregate could not occur. All antibody was bounded to I^{131}-BSA and the equilibrium in solution is reached

$$I^{131}\text{-BSA} + Ab \rightleftharpoons I^{131}\ \text{BSA-Ab}$$

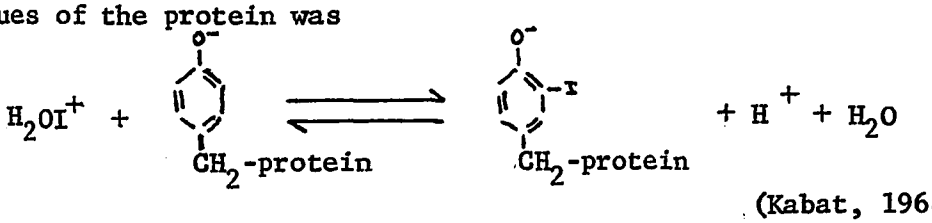
This I^{131}-BSA bound to antibody was found by the radioactivity determinations on the separated precipitation and supernatant formed in 50% SAS. This test gave antigen-binding capacity of the antibody rather than the amount of antigen or antibody spontaneously precipitated.

A. Iodination of BSA

The BSA was labelled with I^{131} by the method described by

Greenwood (1963). The iodination was through an oxidation process with Chloramine T, and sodium metabisulfite was used to stop the oxidation process.

The iodine reacted was H_2OI^+ to iodinate the tyrosine residues of the protein was



(Kabat, 1968)

The column (size about 1 x 30 cm) was prepared by using 1.25 gm Sephadex G-75 which suspended in about 300 ml of borate buffer, pH 8.2, kept overnight at 4°C. To minimize the loss of I^{131}-BSA which sometimes tended to bind with sephadex, 20 mg BGG/ml of borate buffer was allowed to elutriate through the column first.

All the reagents were freshly prepared in phosphate buffer pH 7.6 and the reaction was carried out at room temperature. Five hundred mg of BSA in 0.1 ml was added to I^{131} (0.5-1 Mc.) in a small vial. Then, 4 mg chloramine T in 0.1 ml was added and mixed gently. After one minute, 10 mg sodium metabisulfite was added to stop the reaction. 0.2 ml KI (10 mg/ml) was used for washing the walls of the vial.

The preparation of I^{131}-BSA from the reaction mixture was carried out by gel filtration with a column of sephadex G-75. The mixture was then transferred to the column. The vial was rinsed with another 0.1 ml KI, and the rinse was also put on the column. After the mixture penetrated into the column, borate buffer was added to the

column from the top. Two ml (about 20 drops) of fraction was collected in each of 16 tubes. All 16 fractions were read for their radioactive counts by using a gamma scintillation counter (ORTEC). The first radioactive peak was that of BSA bound I^{131} while the second one was free I^{131} (Appendix 1). The highest peak fraction was then kep for further use.

B. Standardization of antigen

Since so many samples required measurement and the half life of I^{131} was only 8 days, several batches have to be taken and the difference between batches must be minimized. The standardization of the I^{131}-BSA concentration became very important in the measurement of immune response by determining the antigen binding capacity through all batches. The concentration of the stock I^{131}-BSA obtained from the iodinate fraction was determined by means of the Folin-Ciocalteu reagent. The method used was essentially described by Lowry (1957).

A amount of 0.2 ml of I^{131} stock solution was mixed with 2.0 ml of a reagent which was a mixture of 50 ml of 2% Na_2CO_3 in 0.1N NaOH and 1 ml 0.5% $CuSO_4$ $5H_2O$ in 1% sodium citrate. The solution was allowed to stand for 10 minutes at room temperature. Two-tenths ml of Folin-Ciocalteu reagent (1 N in acid) was pipetted rapidly into the mixture with thorough mixing and the extinction at 750 mu. It was ready for reading after standing for 30 minutes.

The concentrations of I^{131}-BSA from 0.125 ug/ml to 12.5 ug/ml were suggested for this method by Farr (1958). It was decided to chose 0.2 ug/ml of I^{131}-BSA for the present study. The known amount of I^{131}-BSA in stock solution in which the protein concentration was determined

by the Folin-Ciocalteu reagent method, was diluted with 1% normal pig serum into 0.2 ug/ml with about 30,000 to 60,000 CPM which was used as a standard for the present study.

C. Reagents for Farr technique

1. Borate Buffer (pH 8.3 ionic strength 0.1) needed in these experiments was made of 6.184 gm boric acid

9.536 gm sodium tetraborate (borax)

4.384 gm sodium chloride

make up to 1 litter, pH 8.3-8.4

2. Saturated ammonium sulfate (SAS)

SAS was prepared and stored at $4^{o}C$ with crystals remaining in the stock SAS to prevent the solution from becoming supersaturated. The solution was filtered and the pH neutralized before being used, since the fractional precipitation is markedly affected by pH.

3. 50% saturated ammonium sulfate (50% SAS)

It was made of one part of SAS to one of borate buffer.

4. Normal pig serum (NPS)

Pooled normal pig serum was heated at $56^{o}C$ for 45 minutes in a water bath. After centrifuged, it was stored at $-20^{o}C$ for further use. Antigen was diluted with 1:100 NPS in borate buffer. In order to prevent denaturation of the I^{131}-BSA by the effects of dilution, 1:10 NPS in borate buffer was prepared for antiserum dilution.

5. Trace labelled I^{131}-BSA

The stock I^{131}-BSA was diluted with 1:100 NPS to the exact concentration desired for the test antigen. Two tenths ug

I^{131}-BSA per ml was used in all the experiments.

6. Trichloroacetic acid (TCA)

Twenty percent aqueous solution.

D. Experimental procedures

1. Antiserum dilution

The preliminary antiserum was 1:10 with borate buffer and subsequent twofold dilution with 1:10 NPS-borate. Each dilution was prepared in two duplicate sets. There were three doubling dilutions of antiserum and each tube contained 0.5 ml aliquots.

2. Controls for each experiment

Normal serum (NPS) control: Three tubes contained 0.5 ml aliquots of normal pig serum (1:10). These tubes received both I^{131}-BSA and SAS.

TCA control (protein-bound I^{131}-controls): Three tubes added 0.5 ml aliquots of normal pig serum (1:10). These tubes received TCA, but not SAS.

Antigen-added controls: Three tubes contained 0.5 ml aliquots of I^{131}-BSA dilution only.

Batch control: One sample from the preliminary experiment which had shown high anti-BSA titer, was used for the determination of antigen binding capacity, there were two duplicate vials of the same sample in each batch as a control for measuring the differences among batches.

3. Experimental procedure of Farr method

Using a volumetric pipette, 0.5 ml of the I^{131}-BSA dilution was pipetted into all experimental and control tubes. The tubes were thoroughly mixed immediately after antigen was added.

All tubes were incubated overnight at 4°C.

Following overnight incubation, 1.0 ml SAS was added to all experimental tubes and normal serum control tubes only. Mixed well with mixer immediately after added SAS. After the addition of SAS, the tubes were incubated at 4°C for 30 minutes, then centrifuged for 30 minutes at 4°C in a refrigerated centrifuge at 2,000 rpm. The supernatants were decanted and discarded. The tubes were blotted on absorbant tissues to take up the remaining drops on the edge of the tubes. After the precipitates were washed with 3 ml of cold 50% SAS and mixed to resuspend the precipitates, the tubes were immediately centrifuged at 4°C for 30 minutes at 2,000 rpm. The supernatants were decanted and blotted as previously.

Antigen-added controls were used as a counting standard. TCA controls were added 1.0 ml of 20% TCA and centrifuged for 30 minutes at 2,000 rpm in a refrigerated centrifuge. Supernatants were decanted and discarded.

4. Counting and calculating procedure

All control and experimental tubes were counted by a gamma scintillation counter (ORTEC). Radioactivity was determined as counts per minute (CPM).

The percent antigen bound in each experimental and batch control tubes were obtained by the following calculations: (Farr, 1970).

A. Determine the percent protein-bound antigen:

$$\% \text{ antigen-bound antigen} = \frac{\text{cpm in TCA ppt}}{\text{cpm in Ag-add control}} \times 100$$

B. Calculation of correction factor (supernatant)

$$C.F. = \frac{\text{cpm in ppt (precipitated)}}{\text{cpm in TCA ppt -cpm in NPS control}}$$

C. Corrected supernatant cpm

= (TCA- average ppt cpm) x C.F.

D. % antigen in supernatant = $\frac{\text{corrected supernatant}}{\text{TCA}}$
(% antigen not bound)

E. % antigen bound (precipitated) = 100% - % antigen not bound

3.6.4 Characteristic of observation

The percentage of antigen bound by antibody was estimated by the radioactivity as described earlier. There were six counts which came from two duplicate sets, each with three dilutions. A regression line of percent antigen bound was computed on the logarithmic dilution factor based on which the percent antigen bound of each sample was estimated at the 1:10 dilution (Rathbun, 1970). The conventional approach by plotting the percent antigen bound against the reciprocal of the dilution of the sample on a semilog paper would produce the same results. In regression analysis the percent antigen bound was on the linear scale and the reciprocal of dilution used on the logarithm (Jones, 1976). The estimate represented the measure of immune response level in a pig. In addition to the immune response level expressed as percent antigen bound, information on sex, birth weight, and weaning weight of each pig was also recorded.

3.7 Statistical methods

The statistical methods used in the present study consisted of

analysis of variance and co-variance, regression analysis and path analysis. All analyses were carried out on the IBM 370/158 computer using the generalized LS program available at the Department of Genetics (Mi, Onizuka and Wong, 1977).

3.7.1 Adjustment, transformation and distribution of data

The data obtained from the batch used as a control were analyzed by the one-way analysis method. The differences of the overall mean and each batch mean were used to adjust for batch differences.

The angular transformation which was developed for binomial proportion was used to transformed the data before further analyses. The computation procedures were illustrated by Snedecor (1967). The histogram of the distribution of samples was given by two different scales, namely percentage and angle. Tests of normality, skewness and kurtosis for the distribution in two different scales were carried out according to Snedecor (1967). Since the most of statistical techniques were based on the assumption of normal distribution of sample, the data which had the best fit in a normality test would be chosen for analyses.

3.7.2 Mathematical models used in data analyses

In any analysis it is important that the mathematical model underlying the analysis and the assumptions made in using the model be well known. Several general models which were discussed by Harvey (1960) were used in the present study.

1. One-way classification model

$$Y_{ij} = \mu + \Sigma a_i + e_{ij}$$

where Y_{ij} = j^{th} observation in the i^{th} A class (the A classes may represent sex, or birth weight or weaning weight or daily gain, or immune response at a specific stage, etc.)

μ = the overall mean

a_i = the effect of the i^{th} A class expressed as a deviation from the overall mean

e_{ij} = random error which are assumed to be independent

2. One way classification with regression model

$$Y_{ij} = \mu + \Sigma a_i + b(x_{ij} - \bar{x}) + e_{ij}$$

where Y_{ij} = the j^{th} observation in the ith A class

μ = overall mean

a_i = the effect of the i^{th} A class

b = simple or partial regression of the dependent variable (Y) on the independent continuous variable (x) holding the discrete variable (the a_i) constant

x_{ij} = the continuous independent variate for the corresponding Y_{ij} observation

$\bar{x}$ = the arithmetic mean of the x_{ij}

e_{ij} = the random errors

An alterable model without Σa_i class could be used only for linear regression.

3. Two-way classification with interaction model

$$Y_{ijk} = \mu + a_i + b_j + (ab)_{ij} + e_{ijk}$$

where:

Y_{ijk} = the k^{th} observation in the jth B class and i^{th} A class

μ = the overall mean

a_i = effect of the i^{th} A class

b_j = effect of the j^{th} B class

$(ab)_{ij}$ = effect of the ij^{th} AB subclass after the average effect of A and B have been removed. These are the individual interaction effects expressed as a deviation from the mean μ.

e_{ijk} = random errors

4. Nested classification model

$$Y_{ijk} = \alpha + a_i + b_{ij} + e_{ijk}$$

where

Y_{ijk} = the k^{th} observation in the j^{th} B class within i^{th} A class

α = the overall mean when equal frequencies exist in all subclasses

a_i = effect of the i^{th} class

b_{ij} = effect of the j^{th} B class within i^{th} A class

e_{ijk} = random errors

3.7.3 Estimation of the variance component

The variance components due to between sires, between dams within sire and within dam were analyzed by 2-stage nested analysis of variance and estimated with the expected mean square as showed below:

Source	Expected mean square
Between sires	$\sigma_w^2 + K_2\sigma_d^2 + K_3\sigma_s^2$
Between dams/sires	$\sigma_w^2 + K_1\sigma_d^2$
Within dam	σ_w^2

where σ_w^2 is variance attributable to differences among progeny of the same female, σ_d^2 is variance attributable to differences among the progeny of females mated to the same male, and σ_s^2 is variance attributable to differences among progeny of different males. K_i's are the coefficient of variance component which were calculated according to the method given by Gates and Shine (1962). The same is true for covariance. The coefficients K_1, K_2, and K_3 were determined by the formulas below:

$$K_1 = (n.. - \frac{\Sigma_i \Sigma_j n_{ij}^2}{n_{i.}}) \text{ / d.f. (dams)}$$

$$K_2 = (\frac{\Sigma\Sigma_{ij} n_{ij}^2}{n_{i.}} - \frac{\Sigma\Sigma_{ij} n_{ij}^2}{n..}) \text{ / d.f. (sires)}$$

$$K_3 = (n.. - \frac{\Sigma_i n_{i.}^2}{n..}) \text{ / d.f. (sires)}$$

According to Hazel, Baker and Reinmiller (1943), the variance of immune response (P) was attributed to a genetic variance (G), a litter environment or between dam environment (L) and a residual environmental factor or within litter environment (E). These sources of variance were additive and statistically independent. Thus the relationship could be shown as follows:

$$V(P) = V(G) + V(L) + V(E)$$

and

$$COV(P) = COV(G) + COV(L) + COV(E)$$

These variances, V(G), V(L) and V(E) could be estimated by using the corresponding component of variance according to the methods which were given by Falconer (1964):

$$V(G) = 4\sigma_s^2$$

$$V(L) = \sigma_d^2 - \sigma_s^2$$

$$V(E) = \sigma_w^2 - 2\sigma_s^2$$

and

$$COV(G) = 4\ COV(s)$$

$$COV(L) = COV(d) - COV(s)$$

$$COV(E) = COV(w) - 2\ COV(s)$$

The relative importance of genetic and environmental components of phenotypic variance then could be easily estimated and were expressed as a ratio of each variance component to the total of variance.

3.7.4 Using path analysis techniques for estimation of the variance components

The quantitative importance of each source in causing variation of immune response was estimated using Wright's (1934) method of path coefficients. The relationship between each source at different stages of immunity development was indicated by correlation coefficients. The calculation for path coefficient and for correlation coefficient of genetic, environmental and phenotypic components were carried out according to the method illustrated by Hazel, Baker and Reinmiller (1943).

3.7.5 Estimation of heritability

Because of the nature of the data available in the presented study, the component of variance from sib analysis was used for estimation of heritability which was discussed fully by Falconer (1964).

$$h_s^2 = \frac{4\sigma_s^2}{\sigma_s^2 + \sigma_d^2 + \sigma_w^2}$$

$$h_d^2 = \frac{4\sigma_d^2}{\sigma_s^2 + \sigma_d^2 + \sigma_w^2}$$

$$h_{s+d}^2 = \frac{2(\sigma_s^2 + \sigma_d^2)}{\sigma_s^2 + \sigma_d^2 + \sigma_w^2}$$

The precision of the estimate of heritability would be determined by the standard error of heritability which was estimated according to the method of Dickerson (1959):

$$\text{S.E. of } h_s^2 = 4^2 \text{ Var } \sigma_s^2 / (\sigma_s^2 + \sigma_d^2 + \sigma_w^2)^2$$

$$\text{S.E. of } h_d^2 = 4^2 \text{ Var } \sigma_d^2 / (\sigma_s^2 + \sigma_d^2 + \sigma_w^2)^2$$

$$\text{S.E. of } h_{s+d}^2 = 2^2 [\text{Var } \sigma_s^2 + \text{Var } \sigma_d^2 + 2 \text{ Cov } (\sigma_s^2\ \sigma_d^2)] / (\sigma_s^2 + \sigma_d^2 + \sigma_w^2)^2$$

where

$$\text{Var } \sigma_s^2 = \frac{2}{K_{ss}^2} \left(\frac{(MS)_s^2}{N_s} + \frac{(MS)_d^2}{N_d} \right)$$

$$\text{Var } \sigma_d^2 = \frac{2}{K_{dd}^2} \left(\frac{(MS)_s^2}{N_d} + \frac{(MS)_w^2}{N_w} \right)$$

$$\text{Cov } (\sigma_s^2\ \sigma_d^2) = \frac{K_{ds}}{K_{ss}} \left(\sigma_d^2 - \frac{2F^2}{N_f\ K_{dd}^2} \right)$$

CHAPTER 4

RESULTS

4.1 Preliminary results

4.1.1 Trial 1: Level of antigen

Antibody was not detectable in blood samples collected at 7 and 14 days after immunization in any of the 15 pigs by either gel diffusion or the passive hemagglutination method. Results for three subsequent samples are given in Table 4-1 showing variation among litters and among doses of BSA. The antibody was scarcely detectable in pigs from the third litter. Most pigs which received 0.5 mg did not have antibody even at 35 days after the first injection. Response to higher doses at 5 mg and 50 mg were good.

4.1.2 Trial 2: Kinetics of immune response

The kinetics of immune response to BSA for each individual pig is displayed in Figure 4-1. The relationship between antibody level as measured in percent antigen bound and days after immunization was well represented by a fourth degree polynomial regression curve for each animal. Variations among individuals were apparent. When data were pooled, the mean response curve showed that antibody was not detectable until 6 days. One of the five pigs responded at approximate 2 weeks. Antibody level increased up to 14 days at which time a booster was given. The response curve continued to rise at a decreasing rate. A plateau was reached approximately at 35 days.

4.1.3 Planning for the main experiment

Based on the above results, two dose levels of BSA were chosen,

Table 4-1. Effects of BSA Dose Level and Time Lapse Since Immunization

Litter number	Pig number	0.5 mg						5 mg						50 mg					
		21		28		35		21		28		35		21		28		35	
		G	P	G	P	G	P	G	P	G	P	G	P	G	P	G	P	G	P
1	1	0	0	0	0	0	0												
	2	0	0	0	x	x	x												
	3							0	x	x	x	x	x						
	4													x	x	x	x	x	x
	5													x	x	x	x	x	x
2	6							x		x	x	x	x						
	7							x		x	x	x	x						
	8													x		x	x	x	x
	9													x		x	x	x	x
3	10	0	0	0	0	0	0												
	11	0	0	0	0	0	0												
	12							0	0	0	0	x	x						
	13							0	0	0	0	0	x						
	14													0	0	0	0	x	x
	15													0	0	0	0	0	x

21, 28 and 35 are days after immunization
G=gel diffusion, P=passive hemagglutination
0=no response, x=response

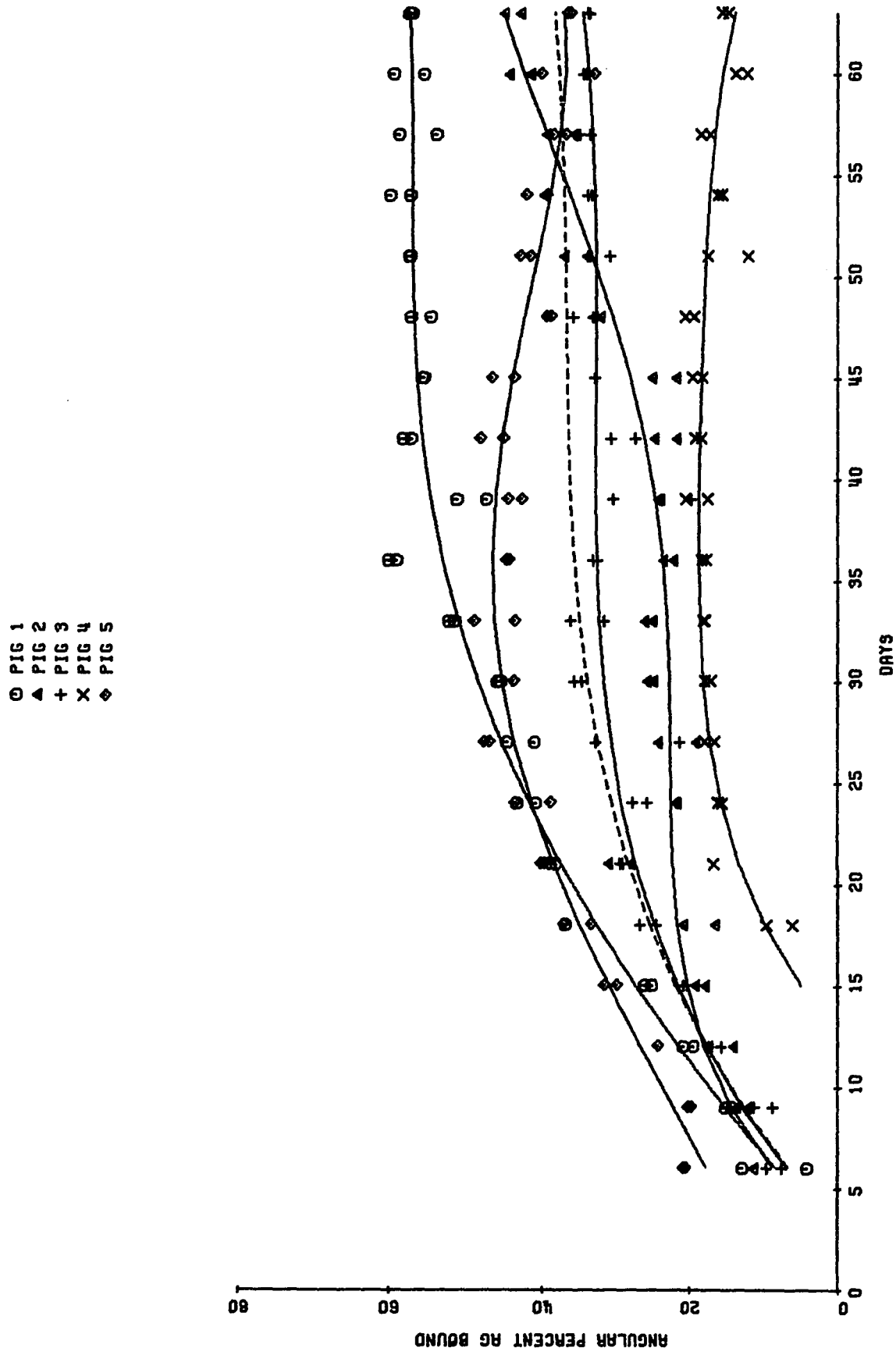


Figure 4-1. The Kinetics of Immunological Response to BSA in Pig

namely 5 and 50 mg. Since the early weaning at 28 days of age and subsequent separation by litter were established practices in the herd, it was necessary to select the weaning time for immunization. It was shown that a good response would be expected 2 weeks later. Therefore the earliest possible time for antibody determination was at 42 days of age. Because of a scheduled hog cholera vaccination at 7 weeks of age for all pigs, it was decided to take blood samples at 14, 21 and 42 days after immunization which corresponded to 42, 49 and 70 days after birth. The first sample would measure the primary response, Rp, since a booster injection was given immediately after drawing blood. The second sample was taken seven days later but prior to hog choloera vaccination, in order to minimize possible interactions between antigens. The determination of antibody level in the second sample was assumed to reflect the secondary response, Rs, The third sample would represent the peak level of antibody production, Rm.

4.2. Data description

Two hundred eighty-one and 266 pigs were immunized with 5 mg and 50 mg of BSA, respectively. Complete information for sufficient data for analysis was 241, 254 and 235 pigs for the dose of 5 mg of BSA at the three stages of immune response, respectively. The corresponding number of pigs in 50 mg of BSA was 204, 204 and 200. The causes of loss of sample are given as follows:

Causes of loss	5 mg			50 mg		
	Rp	Rs	Rm	Rp	Rs	Rm
Death of pig	10	11	13	13	13	15
Insufficient quantity of blood for analysis	4	4	6	6	7	7
Lost during blood processing or centrifuge	6	3	5	6	5	6
Vial broken during thawing	5	4	6	7	5	7
Contamination	9	3	8	12	11	13
Limitation in the method of detection	3	1	3	15	15	14
Other accidental causes	3	1	5	3	6	4
Total	40	27	46	62	62	66

4.3 Data adjustment and transformation

Analysis of all control samples, two for each batch, indicated that there were highly significant differences ($p < 0.01$) among the 18 batches. These differences did not display any time trend and were assumed to reflect random errors.

There were a total of 458 pigs, 254 of which received a dose of 5 mg of BSA and 204 of which received 50 mg of BSA. The mean antibody level under the dose of 5 mg as measured by percent antigen bound was 44.3, 68.9 and 79.1 for Rp, Rs and Rm, respectively. The corresponding means were 16.2, 18.8 and 30.1 under the dose level of 50 mg of BSA.

The distribution of individual response within dose level was not normal. The departure from normality was found to be statistically

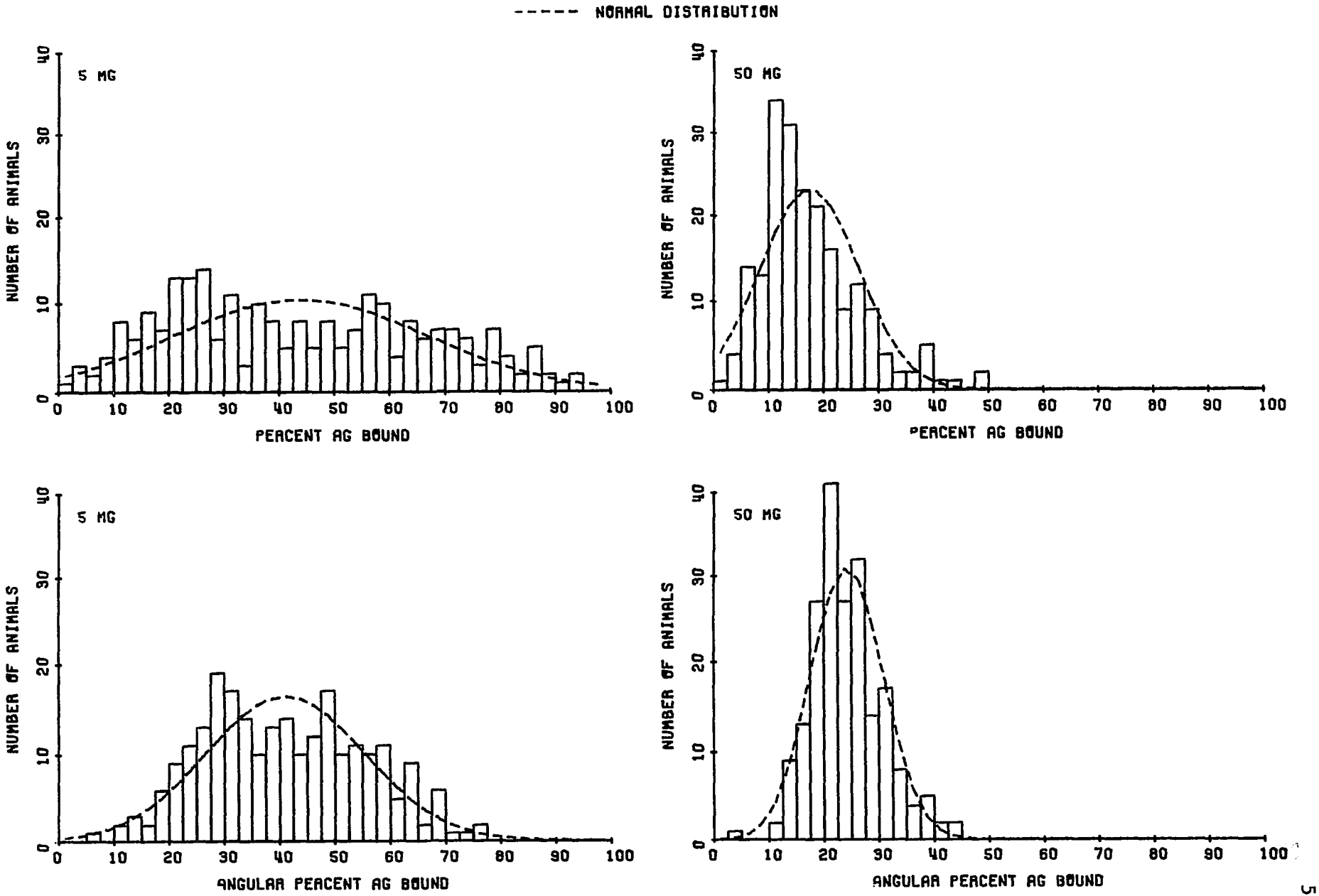


Figure 4-2. Distribution of Immunological Response to BSA at Day of 14 After First Immunization

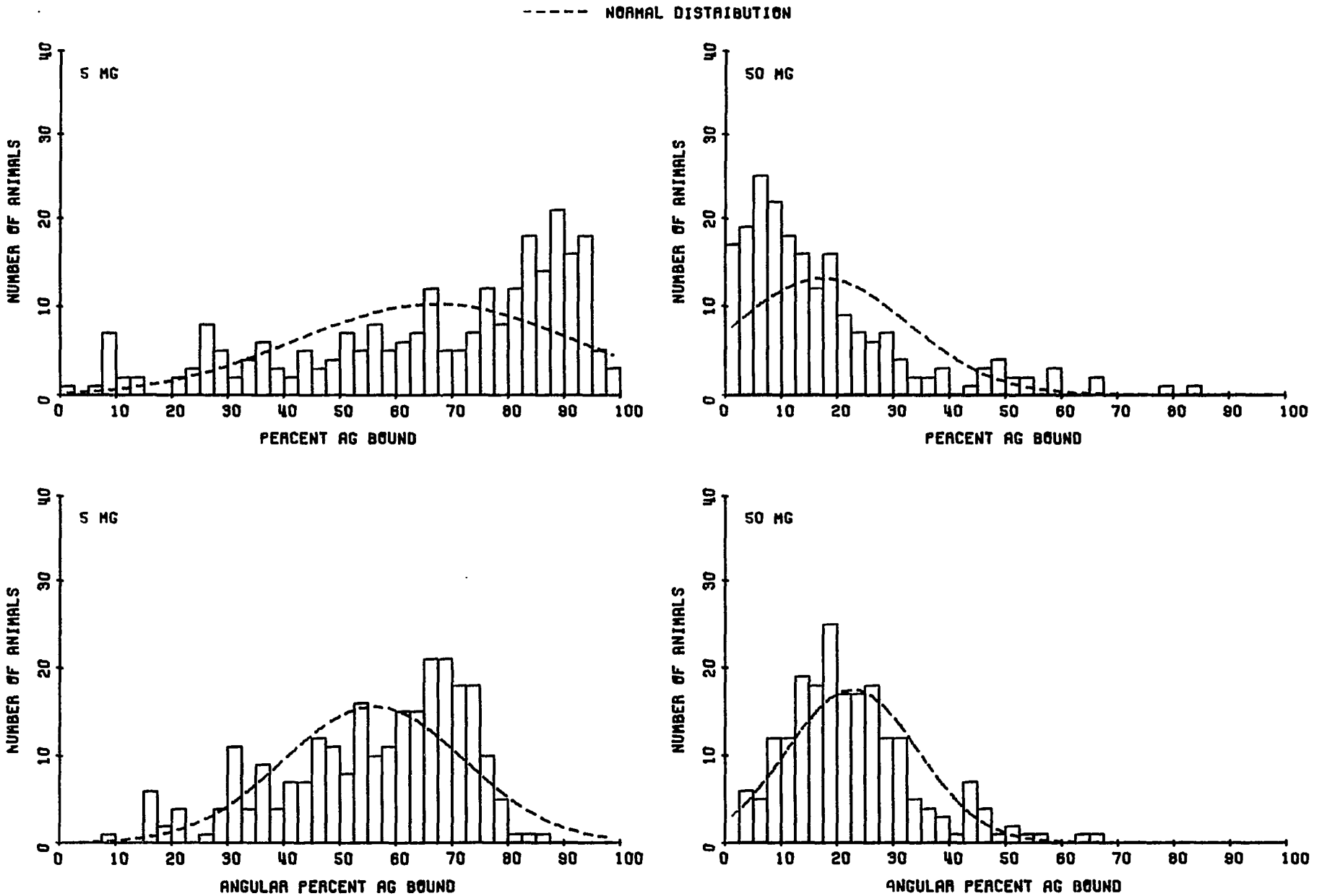


Figure 4-2. Distribution of Immunological Response to BSA at Day of 21 After First Immunization

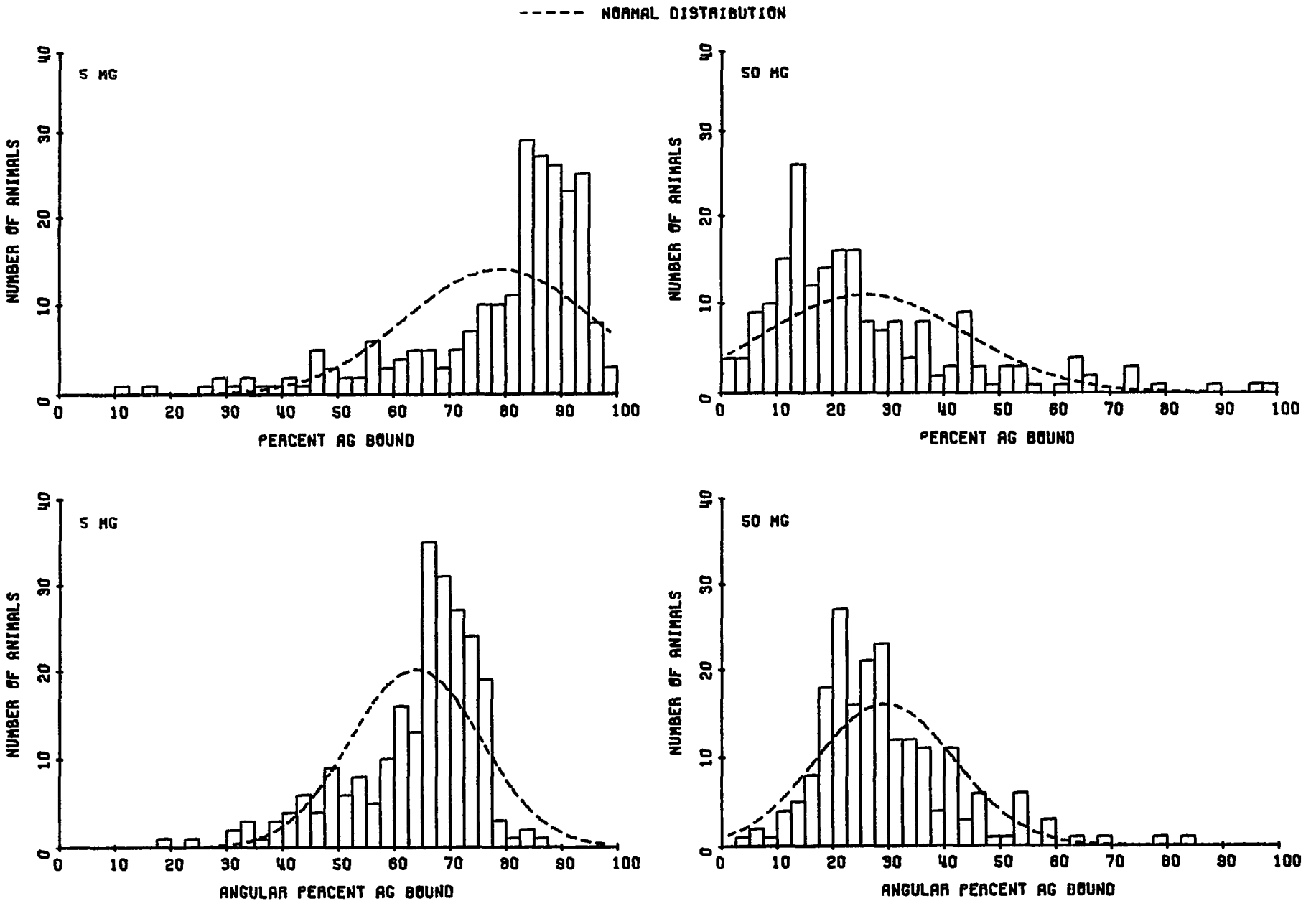


Figure 4-2. Distribution of Immunological Response to BSA at Day of 42 After First Immunization

significant ($p < 0.01$). The approximation of normality by angular transformation of the data is clearly demonstrated in Figure 4-2 to 4-4. The transformed values expressed in degree were used for analysis.

4.4 Effects of sex, dose level and body growth on immune response

Because there were highly significant differences among litters, data were analyzed on a within litter basis using model 2 in which effects of sex, dose level, birth weight, weaning weight or preweaning daily gain on response were tested. None of the factors were found to be important except the dose level of BSA ($p < 0.01$). Therefore, data would not be adjusted by sex, birth weight and weaning weight. Pigs which received 5 mg of BSA demonstrated a consistently higher response than those receiving 50 mg of dose level in all three response measurements, i.e. Rp, Rs and Rm (Table 4-2).

The mean response curves for the two dose levels are shown in Figure 4-5. For pigs which received 5 mg of BSA, there was a marked increase in antibody level between the primary and secondary response. The increase presumably continued at a reduced rate until a plateau was reached. A dose of 50 mg of BSA produced significantly lower primary response. Antibody level seemed to increase slightly after a booster injection as shown by the peak response measured at 42 days after the first immunization.

4.5 Interaction between the dose level and litter

The interaction between the dose level and litter was tested using model 3. The results are given in Table 4-3. There was no evidence of interaction in any of the three responses. Highly significant differences were found between two dose levels and among

Table 4-2. Effect of Dose on Immune Response

Response	Mean response (%)		
	5 mg	50 mg	difference
Rp	44.34	16.18	28.16**
Rs	68.96	18.76	50.20**
Rm	79.09	30.09	49.00**

** $p < 0.01$ highly significant

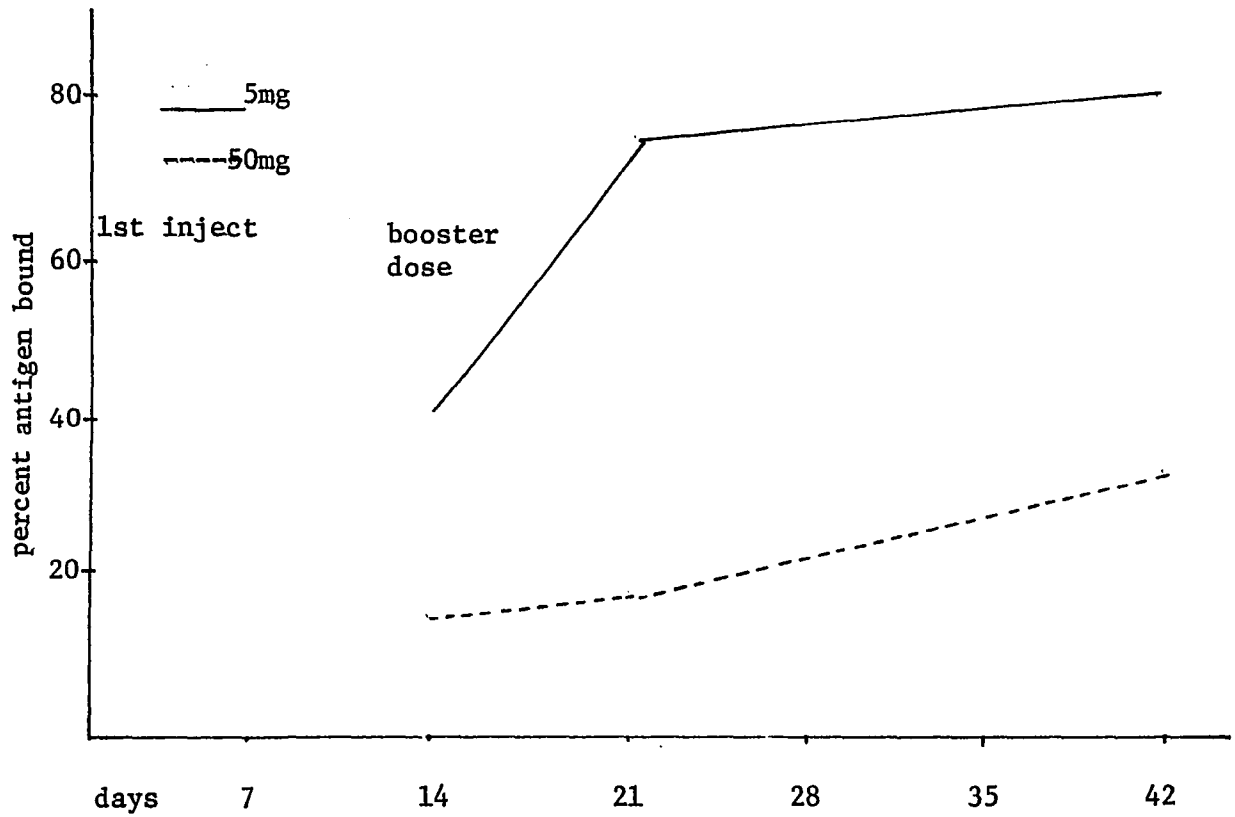


Figure 4-5. Effects of dose level on the immune response in the three stages of immunity development.

Table 4-3. Effects of Dose Level, Litter and Their Interaction

Sources	DF	Mean square		
		Rp	Rs	Rm
Dose level	1	7758.77**	33722.10**	29867.70**
Litter	23	338.75**	693.13**	872.34**
Interaction	23	79.62	156.55	215.50
Residual	111	77.30	153.96	136.76

** $p < 0.01$ highly significant

litters in all responses. The results indicated that the effects of dose level and litter were additive, high responders remaining high in both levels.

4.6 Intrarelationship of immune response measured at three stages

The effect of early antibody production on subsequent response was analyzed by the regression method (model 2) on a within litter basis. Estimates were derived for each dose level separately. As shown in Table 4-4, all responses were found positively correlated. The primary response accounted respectively for 48 and 26 percent of the variation in the secondary and peak response in simple regression for pigs receiving 5 mg of BSA. The secondary response contributed 45 percent of the variation in the peak response. Similar relationships among responses were observed in pigs treated with 50 mg of BSA. When the primary and secondary response were considered simultaneously, the influence of the former on the peak response became nil. The path diagram (Figure 4-6) shows that effect of the primary response on the peak response, if any, would operate through the secondary response.

4.7 Analysis of variance for immune response

Based on model 4, the total variation of each immune response was partitioned into three sources; namely, between sires, between dams within sires, and within dams within sires (Table 4-5). The difference among sires was found to be statistically significant in immune response under the dose of 5 mg of BSA except in the primary response. Variations among dams within sires were significant only in the primary response.

Table 4-4. Effects of Early Response on Subsequent Antibody Production

	5 mg			50 mg		
	b	b_s	R^2	b	b_s	R^2
Simple regression:						
Rs on Rp	0.798**	0.067	0.48	1.108**	0.122	0.44
Rm on Rp	0.424**	0.058	0.26	0.923**	0.178	0.20
Rm on Rs	0.486**	0.044	0.45	0.738**	0.095	0.36
Multiple regression:						
Rm on						
Rp	0.069	0.069	0.45	0.186	0.212	0.37
Rs	0.445**	0.060		0.665**	0.127	

[1] b = regression coefficient

[2] b_s = standard error of b

[3] R^2 = coefficient of determination

** $p < 0.01$ highly significant

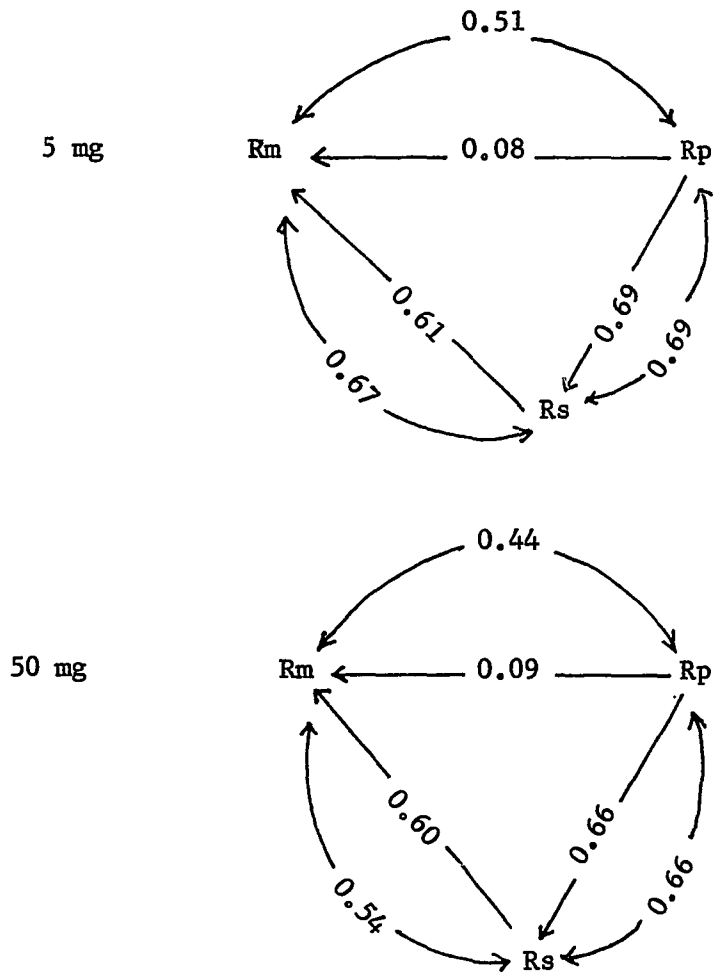


Figure 4-6. Path Diagram Illustrates the Intrarelationship of of Immune Response Measured at Three Stages

Table 4-5. Analysis of Variance and Covariance for Immune Response

Sources	DF	Mean square			Mean cross products		
		Rp	Rs	Rm	RpRs	RpRm	RsRm
				5 mg			
Between sire	17	550.73	655.08**	288.80**	338.80**	182.37*	290.03**
Between dam/sire	45	352.20**	237.42	110.23	147.21	81.38	93.63
Within dam	153	124.26	164.87	86.58	99.25	52.73	80.21
				50 mg			
Between sire	17	99.33	277.24	440.29*	102.63**	99.23**	233.17
Between dam/sire	36	70.92**	228.44**	222.44**	35.74	16.33	151.62**
Within dam	107	23.27	65.36	97.98	25.79	21.47	48.25

* $p < 0.05$ significant

** $p < 0.01$ highly significant

For pigs treated with 50 mg of BSA, no sire difference was detected with the possible exception of the peak response, Rm. However significant differences were found among dams within sires in all three immune responses studied. The lack of significant differences among sires in the primary response might be interpreted to mean that the genetic contribution to the early response as measured at 14 days after immunization was not important. As its variation would be expected to manifest mainly environmental influences, this part of data was not used for the estimation of genetic parameters.

4.8 Estimates of variance component for immune response

In Table 4-6, the three variance components were derived from the mean squares in the analysis of the variance table given above. These were attributed to (1) differences among sires, σ_S^2; (2) differences among dams, σ_D^2; and (3) differences among litter mates, σ_W^2. The subscript "i" was used to identify the three immune responses. Since all three response measurements were used, the components of covariance, σ_{SiSi}, σ_{DiDi}, and σ_{WiWi}; were calculated by the same procedure. The genetic variance, σ_G^2; litter environmental variance, σ_L^2; the individual environmental variance, σ_E^2; and the phenotyic variance, σ_P^2; were obtained based on the procedure outlined under materials and methods. These estimates are given in Table 4-7. The genetic variance was a measure of the average effects of all genes on the immune response. The litter environmental variance represented the effects of environmental factors which influenced the litter, and the individual environmental variance was the residual environmental factors composing the effects of dominance, espistasis and environmental factors peculiar

Table 4-6. Component of Variance and Covariance of Immune Response

Response	$\sigma^2 S$	$\sigma^2 D$	$\sigma^2 W$	Combination	$\sigma S_i S_i$	$\sigma D_i D_i$	$\sigma W_i W_i$
Rp	15.24	67.92	124.26	RpRs	15.76	14.29	99.25
Rs	34.24	21.62	164.87	RpRm	8.29	8.54	52.73
Rm	14.83	7.05	86.58	RsRm	16.37	4.00	80.21

$\sigma^2 S$ = between-sire component

$\sigma^2 D$ = between-dam, within-sire component

$\sigma^2 W$ = within-progeny component

$\sigma S_i S_i$ = covariance of sire component between two stages

$\sigma D_i D_i$ = covariance of dam component between two stages

$\sigma W_i W_i$ = covariance of progeny component between stages

Table 4-7. Genotypic and Environmental Variance and Covariance for Immune Response

Sources		Variance (σ^2)		Covariance (σ)
Genotypic	G_1	60.95	G_1G_2	63.04
	G_2	138.20	G_1G_3	33.16
	G_3	59.28	G_2G_3	65.48
Litter environment	L_1	52.24	L_1L_2	-1.47
	L_2	0	L_1L_2	0.25
	L_3	0	L_2L_3	-12.37
Individual environment	E_1	93.78	E_1E_2	67.73
	E_2	95.77	E_1E_3	36.15
	E_3	56.94	E_2E_3	47.47
Phenotypic	P_1	206.97	P_1P_2	129.30
	P_2	233.97	P_1P_3	69.56
	P_3	116.22	P_2P_3	100.58

Subscripts 1, 2 and 3 are designed for Rp, Rs and Rm, respectively.

to individual pigs. The relative importance of these variances as each expressed in percent of the total phenotypic variance is illustrated by Table 4-8. The genetic variance of the three immune response were 29, 59 and 51 percent of the phenotypic variance showed an important in secondary and peak response. The litter environment decreased in importance from 25 percent of the phenotypic variance in primary response to zero in the secondary and peak response. The individual environmental variance in the three immune response showed a stable variation (45%, 40% and 49% for the Rp, Rs and Rm) among the three immune response.

4.9 Path analysis

Genetic and environmental correlation between the three immune responses were analyzed by the method of path analysis. In Figure 4-7, the path coefficients were shown in straight line and the correlation coefficients were given in curve line. The heredity played a significant role in determining variations in immune response particularly in the secondary and peak response. The individual environment showed fairly constant effects on the variation in immune response, while the litter environment showed some influence in the primary response but none in subsequent measurements. The genetic correlations between the two adjacent measurements, i.e. Rp and Rs, and Rs and Rm were considerably higher than that between Rp and Rm. However, the genetic correlation between the secondary response, Rs; and peak response, Rm; was the highest being 0.72. The observation that correlations between the two adjacent measurements were generally higher was also found in individual environment and phenotypic measurement. Since the variance

Table 4-8. The Percent of the Phenotypic Variance in Response Attributed to Genetic, Litter and Individual Environment

Sources		Percent
Genetic	G_1	29.45
	G_2	59.07
	G_3	51.01
Litter environment	L_1	25.24
	L_2	0
	L_3	0
Individual environment	E_1	45.31
	E_2	40.93
	E_3	48.99

Subscripts 1, 2 and 3 are designed for Rp, Rs and Rm, respectively.

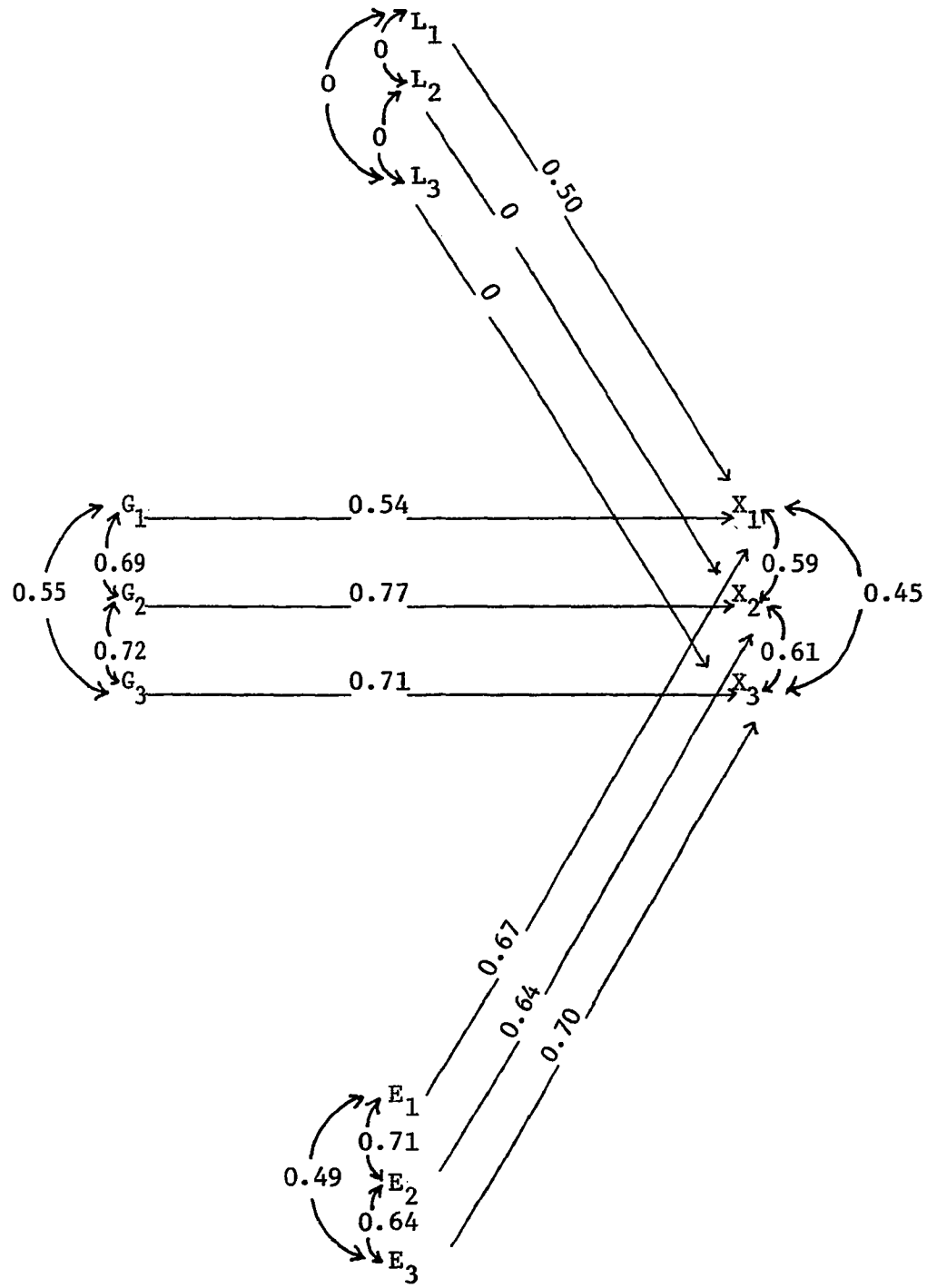


Figure 4-7. Path Diagram of Genetic and Environmental Relations in Immune Response

components of litter environment for the secondary and peak response were estimated to be zero, correlations between two response measurements were assumed to be negligible. The heritability estimate derived from the path coefficient squared was 0.29 for primary response, 0.59 for secondary response, and 0.51 for peak response. There were 71, 41 and 49 percent of the observed phenotypic variation remained and attributed to environmental sources.

4.10 Heritability estimates

The heritability of immune response could be estimated from sire component, dam component or the two components combined. These estimates are shown in Table 4-9 with their standard errors. Heritability obtained from the sire component for secondary response and peak response are 0.51 ± 0.07 and 0.40 ± 0.07. These estimates are considered to be more precise than those calculated from other variance components. Due to highly significant differences between dams, the heritability of primary response calculated from the dam component is an overestimate.

Table 4-9. Heritability Estimates of Immune Response

Method of estimation	Rp	Rs	Rm
	$h^2 \pm$ s.e.	$h^2 \pm$ s.e.	$h^2 \pm$ s.e.
S	0.29 ± 0.06	0.63 ± 0.07	0.57 ± 0.07
D	1.13 ± 0.09	0.39 ± 0.06	0.26 ± 0.06
S + D	0.80 ± 0.04	0.51 ± 0.04	0.40 ± 0.04

h^2 = heritability

s.e. = standard error of h^2

S = sire component

D = dam component

S + D = sire and dam component combined

CHAPTER 5

DISCUSSION

The present study was designed to evaluate the effects of genetic and environmental factors on immune response to BSA in pigs. This was the first attempt to use a large mammalian species for the genetic study of immune response. Large extant pig herds can be used to facilitate large-scale experimentation. A mating plan which employs randomly selected breeding animals from the population, one boar to several sows, provides full- and half-sib data for genetic analysis. The phenotypic variation can be partitioned into three sources: (1) heredity, (2) common maternal environment, and (3) individual environment. The large herd selected for the present study is an intensive production operation in which all animals are kept in complete confinement. Feeding and management are maintained constantly and uniformly in the herd.

The use of bovine serum albumin is considered a suitable antigen for study. It is a pure soluble protein with a large molecular weight, 96,000, and is a potent immumogen when used with Freund's adjuvant (Hildemann, 1970). The anti-BSA antibody can be measured by the method of antigen binding capacity (Farr, 1958). The measurement is recorded in percentage and demonstrates a continuous distribution. It is considered more precise than the method of titration which records the dilution factor at the end point of reaction. The antigen binding capacity method used for the measurement of immune response in the present study has been compared with several commonly used methods and was found to be a good method for detecting the primary interaction

between antigen and antibody (Minden, 1965, 1969). This method is sensitive and reproducible (Oakes, 1972) and is considered to be simple for large-scale processing (Chard, 1970). It has also been shown that materials used in the method are relatively inexpensive, and there are fewer problems concerning variation between different batches of reagents. The data in the present study are expressed in percent antigen bound as used previously by Minder (1969), Green (1969), Rathlum (1970) and Jones (1976).

The response of most pigs receiving a dose of 0.5 mg BSA could not be detected up to 35 days after immunization, while animals treated with 5 mg and 50 mg showed measurable response at 21 days or earlier. This low dose of antigen, 0.5 mg, is considered sub-optimal. The result is consistent with the findings of Siskind (1968) in rabbits.

As pointed out by Sang and Sobey (1954), the selection of optimal time for measuring response is critical. It is also important to the definition of phenotype for a genetic study. The kinetics of response to BSA in pigs as studied provides essential information for such a purpose. However, there are many other factors which must be taken into consideration when a large-scale experiment is being conducted in a production herd. For example, it might be better to postpone both primary and secondary measurements to a later stage in the present study. However, the routine vaccination program against hog cholera could have interfered with the response to BSA. This possibility has been demonstrated by the fact that the depression of immune response to one antigen may occur as a result of the injection of a second antigen (Cremer, 1963; Adler, 1964; Radovich and Talmage, 1967).

It has been shown that the concentration of r-globulin in pig serum decreases to a minimum at around 28 days of age and thereafter increases gradually when pigs are kept with their mothers after birth (Jakobsen and Moustgaard, 1950; Rook, 1951; Foster, 1951; and Nordbring, 1957). On the other hand, pigs which are separated from their dams without getting colostrum have shown the capability of immunoglobulin production at an age as early as approximately 2 weeks (Curtis and Bourne, 1973; All and Porter, 1973) but a normal time for active immunization is between 3 to 6 weeks of age (Hoerlein, 1957; Miller, 1962; and Brambell, 1970).

Sex difference in immune response has been reported in mature mice (Sobey and Adams, 1955). Other investigators have shown that sex hormone could influence immune response (Dougherty, 1952; Butterworth, 1967; Eidinger, 1972; Hudson, 1974). In the present study the measurement of three responses is made in pigs at an early age and the last measurement is made at 70 days of age. In pigs sexual maturity is reached at about 5 months. If there is any degree of sexual development at two months of age its influence on immune response would be too small to measure in the present study.

The notion that the rate of body growth of an individual may be related to the development of antibody-producing capability is an interesting one. In the present study birth weight, weaning weight and daily gain in weight from birth to weaning are three measures of early growth and development of an individual. The weight at birth measures prenatal growth and the second measurement represents postnatal growth up to weaning at 28 days of age. The average growth increment

is measured by the total weight gain from birth to weaning divided by the length of the nursing period. However, there is no evidence of an association between early growth of an individual and its ability to develop immune response. No other published data are available for comparison.

There were highly significant differences found between the immune response of both antigen doses in three stages of immune response. It was shown that pigs given 5 mg of BSA had a higher immune response than that of pigs given 50 mg of BSA. These results are in agreement with the findings reported by Eisen and Siskind (1964) and Siskind (1968) in that the high dose of antigen results in a lower serum level of antibody as compared with the lower dose. This effect of the higher dose of antigen can be attributed to induction of immunological tolerance. Soluble serum proteins, such as serum albumin, have shown low dose or high dose tolerance in laboratory animals (Mitchison, 1968). Thus, 5 mg of BSA may be an optimal dosage, while 50 mg of BSA can induce high dose tolerance in 28-day old pigs. This immune suppressive effect of 50 mg of BSA may play a role in the genetic expressivity of the immune response and result in a wide variation among sire groups in the present study.

The immune response increased progressively with time after immunization, and this increase was greater with lower doses of antigen, while the high dose showed a much lower and slower increase in the immune response. With 5 mg of BSA, the secondary response increased rapidly after the booster dose, but the secondary response of 50 mg of BSA showed only a slight increase. These findings are

consistent with the reports of Uhr (1962) and Siskind (1968). The intra-relationships of the three stages of immune response show highly significant correlations in the present study. This is supported by the report of Sobey (1955) who has indicated that there is a high correlation between the two periods studied in the course of the development of immune response. The influence of primary response on secondary response or secondary response on peak response was more important than that of primary response on peak response. These effects were also illustrated by the path diagram in Figure 4-6. Similar results of the intra-relationships of immune response measured at three stages were seen in both doses.

Interaction between dose and litter was not apparent in this present study. This indicates that the litter mates show a consistent capability of immune response in different environments, i.e. a high responder to the treatment of 5 mg of BSA is also found to be high responder to 50 mg of BSA. This result suggests that the effects of interactions between dose and litter can be neglected from the genetic analysis of immune response.

Since the lack of significant differences among sires in immune response to 50 mg of BSA indicated that the genetic contribution to response was not important, this part of the data was not used for the estimation of genetic parameters.

In the analysis of variance for the immune response in the three stages of immunity development for both doses, highly significant differences among sire groups were found in secondary and peak responses but not in primary response after a dose of 5 mg BSA. This suggests

that maternal effects may be involved in the primary response. Thus, the sources of variation in immune response due to the maternal effects could be attributed to variation in the absorption of maternal immunity from the colostrum. Perry (1967a, 1967b) indicated that the maternal effect is a very important source of variation in the efficiency of antibody absorption in pigs. The speed of the elimination of passive immunity from the serum of the pig is different among individuals (Miller, 1962). The influence of passive immunity on the active antibody produced has been demonstrated in the comparison of colostrum-deprived and colostrum-fed pigs in response to some of the antigens (Hoerlein, 1957; Segre, 1962a, 1962b; Kaeherle, 1968; and Kim, 1966). The colostrum-fed pig responded highly and rapidly to the antigen. The pigs showed a higher response to a combine antigen with serum from an older pig than that from a younger pig. This gave evidence that a certain level of immunoglobulin may enhance the production of the antibody (Segre, 1962a, 1962b). The immune-colostrum, interfering with active antibody production in pigs, has been reported by Hoerlein (1957) and Segre (1962a, 1962b). Their evidence suggested a complicated maternal effect on the immune response. The effect was gradually reduced with age; the relative importance went from 25 percent of the total phenotypic variance on the 14th day after injection, to zero on the 21st and 42nd day. Factors which operate to reduce the importance of heredity in early stage of immunity development are apparent.

Path coefficients for individual environment were notably constant in variation among the three stages of immune response. This suggests

that environmental factors, other than those between litters, play a similar role in the three stages of immune response.

Genetic control of the immune response has been demonstrated by using chemically defined antigens with inbred animals, and immune response genes have been proposed in the control of this particular trait (Mores, 1972; Hildemann, 1973; Marx, 1976). Much evidence also supports the multigenic control of immune response (Playfair, 1968; Simonian, 1968; McDevitt, 1965; Gill, 1971; Eichmann, 1971; Merryman, 1975; Fournie, 1976; Steward, 1976 and Gherman, 1976). Selective breeding for high and low immune responder lines in mice to heterologus erythrocytes has shown that multiple genes are involved in controlling this response (Biozzi, 1970; Feingold, 1976). Genetic control of the immune response to an optimal dose of BSA at specific stages was also shown in this present study. In the present study, the heritability of immune response was found to be different between the two dose levels of BSA. No available studies can be used for comparison. The variation of heritability between the three stages of immune response was also showed in the study. Claringbold and Sobey (1957) have reported a large variation of heritability of immune response to sheep red cells between the primary and secondary response. They have suggested that the factors governing response to a primary dose are considerably different from those governing response to a secondary dose. This view supports the results of the present study. Another study on inheritance of immune response to sheep red cells was conducted by Sobey and Adams (1955) who found a variation of heritability between two stages of primary response. Heritability estimates vary with different antigens

used (Sang and Sobey, 1954: Sobey and Adams, 1961) and also with different measurement methods employed (Sobey and Adams, 1961). Unfortunately the series of studies of Sobey, et al. on inheritance of immune response all used microorganisms or xenogenic erythrocytes as antigen source. Battisto (1972) indicated that complex antigens such as microorganisms or xenogenic erythrocytes tended to obscure results and this was especially true when the test for antibody did not differentiate sufficiently between multiple antigens on cell surface. It should be emphasized that the phenotype of immune response must be well-defined and the heritability must be estimated only for that particular phenotype. In general, under an optimal dose and suitable conditions, the immune response to an antigen would be a medium heritable trait according to results of this present study and a realized heritability estimated by Biozzi (1970). The heritability of immune response to 5 mg of BSA was estimated by variance component of sire, dam or sire and dam combined. The best estimate of heritability was decided by the least standard error of each heritability. In the primary response, the heritability estimated from dam component of variance showed a overestimation, i.e. the value greater than 1. There should be no dominant variance in the dam component because if it exists then this dominant variance should also be present at the secondary and peak response stages. Therefore, the overestimation could be explained as being due to the large common maternal environment variance involved. The estimation from sire variance component could be the most precise estimation for primary response.

Because of maternal effects on the immune response, only the secondary and peak responses showed significant differences in genetic

variance among sire groups. The heritability estimates determined from these two stages may give a more reliable estimation than that derived from the primary response. High genetic correlations were found in these two stages of immune response. This indicates that genetic selection for secondary response will affect the peak response. Since the variance components of litter environment for these two stages were estimated to be zero, the litter environment correlations could not be estimated. This result is expected since the importance of litter environment during the immune development decreases with age, especially after weaning.

As previously described, the large maternal effects were involved in the variance between dams. If the immunoglobulin in each experimental pig serum could be detected before injecting the BSA, the influence of passive immunity would be eliminated. But it was impossible to do so with such a study involving a large number of animals.

Due to the complexity of influences on immune response by various environmental factors, one must attempt to minimize the variation of environment by experiment design. However, for further similar studies certain experimental steps have to be considered: (a) The experimental animals should be of about the same age and have active antibody production potency. It is also important to know that no passive immunity interference exists in the animals. (b) The antigens used should be pure and produce only one specific antibody. (c) The phenotype would be defined as one in which immune response is under a predetermined dose of an antigen at a specified time after immunization. Since immune response is strongly dependent on dose as shown in

the present study, it is important to find an optimum dose for the best response for full genetic expressivity. (d) The suitable time for testing may be chosen at a clearly specified phase of response. It can be obtained by a preliminary trial on the kinetics of immune response. (e) Immunization schedule should be standardized by using the same injection routine. (f) The antigen-antibody reaction should be measured by a method which is simple, reproducible and accurate. (g) The results should be transformed by statistical method to a valid scale.

The results of this study will not only enhance the understanding of genetic and environmental effects on immune respone, but can also be used to assist the animal immunization program for animal health. The genetic action model of this quantitative trait can be used as a basis for selecting disease resistant animals (Biozzi, 1971). Further breeding and selection of high immune response animals will decrease the mortality and/or morbidity rate among animals and result in an increase in growth rate and a corresponding improvement in animal production efficiency. Since it is dangerous and costly to expose animals to pathogens for the purpose of selecting the disease resistance in such large animals, the results of this study will provide a means of detecting the propensity for disease resistance by measuring immune response to harmless antigen.

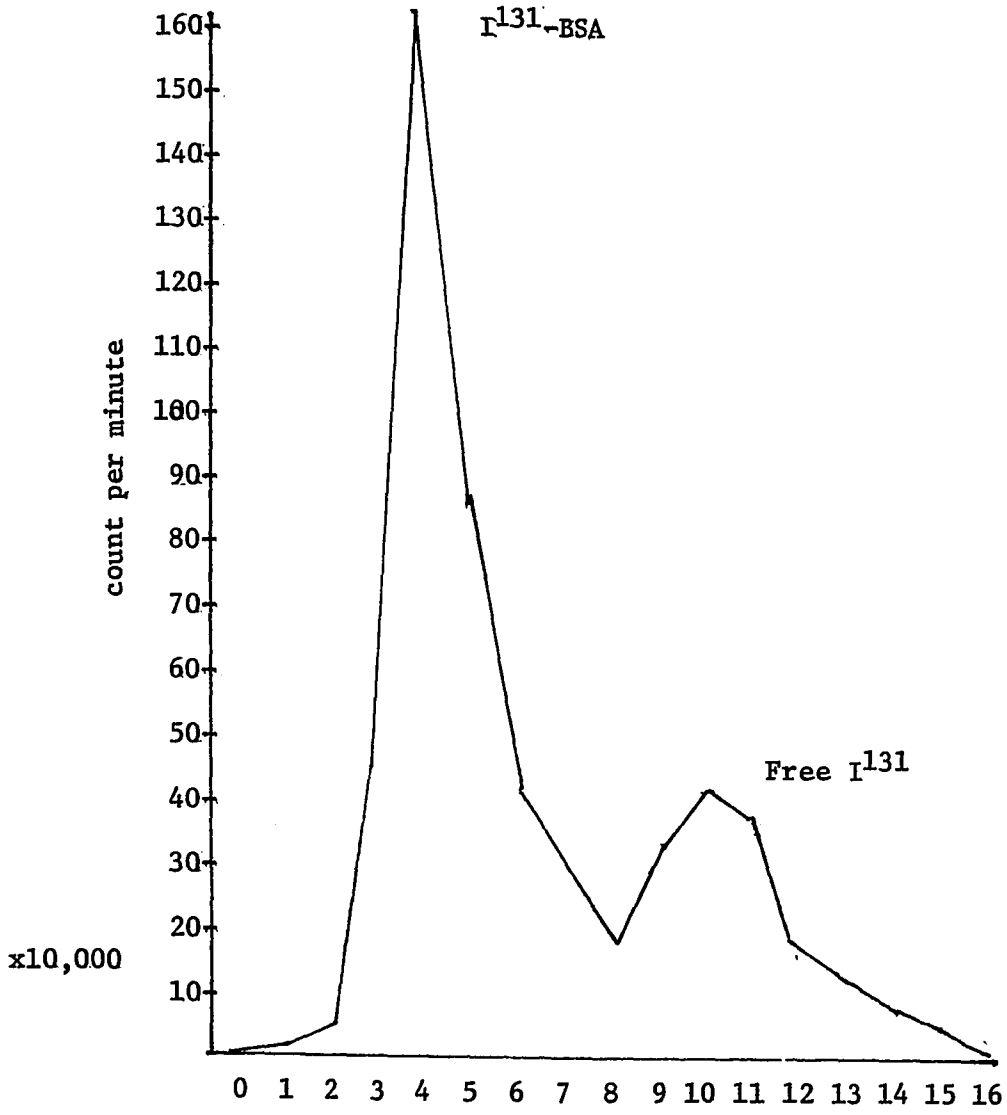
In conclusion, the multigenic control of immune response was demonstrated in the present study.

The genetic and environmental component of variation among three stages of immune response has been fully studied and partitioned into three sources, i.e. genetic variance, litter or common maternal environmental variance, and individual environmental variance. Genetic control of immune response varied among doses and among stages of immune response. Genetic importance was found in secondary and peak responses, while the importance of common maternal environment was only seen in primary response. A constant variation of individual environment was found among the three stages of immune response.

The heritability estimations for secondary and peak responses of 5 mg dose of BSA were found to be 0.51 and 0.40, respectively. According to this heritability, a genetic improvement of immune response would be expected by selective breeding. Genetic correlations for primary and secondary, primary and peak, and secondary and peak responses were 0.69, 0.55 and 0.72. Corresponding values for phenotypic correlations were 0.59, 0.45 and 0.61; for individual environmental correlations, corresponding values were 0.71, 0.49 and 0.64.

No interaction between dose and litter was found in the three stages of immune response, although highly significant difference was seen between doses and among litters.

High dose of BSA, 50 mg, has induced suppression in pigs. The 5 mg dose of BSA would be an optimal dose for immunization in pigs.



Appendix 1. Separation of I^{131}-BSA and free I^{131} by gel filtration with a column of Sephadex G-75

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