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The heritability and characteristics of the time-course of antibody production; a study of viral antibodies in inbred mice.

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THE HERITABILITY AND CHARACTERISTICS OF THE TIME COURSE OF ANTIBODY PRODUCTION

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THE HERITABILITY AND CHARACTERISTICS OF THE TIME-COURSE OF ANTIBODY PRODUCTION: A STUDY OF VIRAL ANTIBODIES IN INBRED MICE

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INTRODUCTION

It has been demonstrated repeatedly that heritable factors markedly influence the response to disease. The operation of genetic factors obeying Mendelian principles has been shown to account for the natural resistance of a number of animal hosts to a variety of pathogenic and toxic agents. Reviews of the genetic aspects of natural resistance and of the host-pathogen relationship have been published by Crew (1), Kozelka (2), Lambert (3), Gowen (4, 5), Parish (6), and Elberg (7).

The studies on natural or inherent resistance deal with the reaction of the animal in its initial contact with the pathogen. Under such circumstances, submission of the host to the pathogen or rejection of the disease occurs within a very short time and evidence is accumulating that this prompt response is not dependent upon antibody of either the induced or passively acquired varieties (8, 9), but is rather due to non-humoral factors. These non-humoral, but genetically controlled factors which function in the response to disease remain at the present time largely unexplained. Although cellular factors, such as number of leukocytes (10, 11), number and location of lymphocytes or macrophages (12, 13, 14) and constitution of the liver and spleen (15); as well as non-cellular factors, such as increased body temperature (16, 17) and high blood pH (18) have been implicated as

the source of natural resistance, all of these remain at the present time only associations and correlations. Genetic proof of their association with disease has not yet been supplied. Preliminary studies which have been done (19) indicate that inbreeding for increased number of leukocytes and high blood pH is accompanied by the loss of the correlation with resistance to disease.

In contrast to the great number of studies on inherent resistance upon first contact with disease, papers on the other factor of resistance, i.e., genetic control of antibody production, have been few in number. Some work has been done with regard to differences between inbred strains of animals in the ability to produce antibody. Lewis and Loomis (20) found that families of inbred guinea pigs differed in their capacity to produce hemolysins to bovine and sheep erythrocytes and agglutinins to B. typhosus and B. abortus. Prigge (21) demonstrated that different strains of guinea pigs had varying responses to diphtheria toxoid. Schütze, Gorer and Finlayson (22) and Gorer and Schütze (23) found strain differences in the ability of mice to produce agglutinins to typhoid bacilli; they were unable to correlate the natural resistance of the strains upon first contact with typhoid and the capacity to produce antibody. In inbred rabbits Lurie (9) showed variation among rabbit strains in terms of antibody production to killed tubercule bacilli and found some correlation with innate resistance. Weiser, Golub and Hamre (24)

found that strains of inbred mice differed in the ability to produce precipitins to egg white and that the strains with high precipitin titers were the most susceptible to anaphylaxis. Gowen (25) vaccinated six inbred strains of mice with inactivated S. typhimurium. The position of the strains relative to one another in terms of survival after challenge was the same in vaccinated and non-vaccinated animals although the vaccinated animals were more resistant to the challenge. He did not, however, measure antibody levels. Fink and Quinn (26) investigated the response of five strains of inbred mice to two different antigens: egg white and pneumococcus polysaccharide. Marked strain differences were found in the antibody titers to each antigen and the comparative ranking of the strains in terms of titer for the two antigens was entirely different. In Davidsohn and Stern's work (27, 28) immune antibody to heterologous red cells was measured in eleven inbred mouse strains. There were definite statistical strain differences in the immune agglutinins and hemolysins to the sheep and chicken erythrocytes employed as antigens; the strain rankings for the production of sheep and chicken antibodies were different. Fink and Rothlauf (29) found differences in strains of mice in the capacity to produce antibody to egg albumin; the antibody level could not be correlated with the susceptibility to anaphylaxis. Ipsen (30, 31) tested immunizability to tetanus toxoid indirectly by noting the survival time after a challenge of toxin. The mouse

strains differed in immunizability, the degree of which bore no relationship to the natural resistance of the strains to tetanus toxin. Mueller, Wolfe, and McGibbon (32) immunized inbred chickens with bovine serum albumin and noted definite differences between the strains in terms of the total amount of circulating antibody nitrogen.

In the works cited above the observed strain differences have been in general attributed to genetic control of antibody production. Although it is true on the basis of these studies to say that antibody production varies with the genetic constitution of the strain of animals employed, the source of these differences may not be attributed, per se, to genetic determinants. It is tempting in the presence of highly homozygous animals with intra-strain titers of small variance and very different inter-strain titers to ascribe the observed differences between strains to the operation of genetic, chromosomal control. However, it is necessary to prove these presumed genetic determinants of antibody production through demonstration of heritability and characterization of the pattern of inheritance in order to rule out associations of a non-chromosomal nature, either heritable, as in cytoplasmic inheritance, or non-inherited such as environmental factors.

The first study attempting to define and characterize genetic control of the production of antibody was that of

Kleczkowski and Kleczkowska (33), who were able to divide rabbits into strong, medium and weak producers of antibody to human serum. The pattern of inheritance strongly suggested the segregation of a single gene, the homozygous gene giving strong antibody production, its homozygous allele weak production and the heterozygous state intermediate production. Schiebel (34) inbred guinea pigs through six generations selecting for either good or absent production of antitoxin to diphtheria. The study showed that the capacity for antitoxin production or non-production was hereditary and although it appeared to represent a simple, non-sex-linked, gene construction, the data were insufficient to define more closely the inheritance mechanism. Sang and Sobey (35) investigated the response of two strains of rabbits and their F_1 offspring to bovine plasma albumin and tobacco mosaic virus. Using the genetic technique of regression of offspring titers to those of the parents (the mean titers of the offspring and parents are plotted on a graph where the log of the parental mean titer is the abscissa and the log of the offspring mean titer is the ordinate; the existence of a straight line joining many such points is taken as evidence of genetic inheritance), he estimated that antibody production to the tobacco mosaic virus was 90 per cent under genetic control. He was unable to demonstrate that genetic factors were important in determining the antibody response to bovine albumin.

Common to these studies of antibody production in

inbred animals after immunization is the determination of the presumably maximum serum titer attained or the mean survival time after challenge. The entire antibody response pattern, from appearance of antibody through its decline, has not been studied in inbred animals, and the probability of inter-strain differences and possible genetic control of the total response pattern also remain to be investigated.

The present study was undertaken to investigate the extent and characteristics of the antibody response in strains of inbred mice, to obtain information about variation of the response pattern with different antigens and to determine the existence, extent and mode of inheritance of possible genetic control of the antibody response. The entire time-course of antibody production was studied, which included: 1) the time of antibody appearance in the serum, 2) the rate of production, 3) the time and amount of maximal antibody response, and 4) the rate of antibody decline. Information about the specificity of response was obtained through the use of two inactivated Type A influenza virus antigens and the measurement of two antibodies to each virus. The curves of antibody production of each antibody to each virus was plotted for each mouse strain and the resulting patterns compared. In addition, a third antigen, a single purified protein antigen was included originally to analyze further the specificity. Finally the inheritance of the observed patterns was studied in the first filial generation of reciprocal matings of the inbred strains.

MATERIALS AND METHODS

Experimental animals: Six strains of mice were employed. Three of the strains: C57BR/cd Jax, AKR Jax and DBA/1 Jax were highly inbred strains obtained from the Jackson Memorial Laboratory, Bar Harbor, Maine; they have been inbred by sibling matings for over 40 generations and thus have reached the theoretical maximal attainable degree of homozygosity (22, 36). These three strains were derived originally from unrelated mice twenty to fifty years ago (37, 38). Two of the strains represented the first filial generation (F_1) of the reciprocal mating of strains C57BR/cd and AKR; the strains were mated in this laboratory from parents supplied by the Jackson Laboratory. The sixth strain was an albino Swiss strain maintained in this laboratory for many years by inbreeding within the strain but without regard for sibling or other selective matings. This latter strain was presumed to consist of somewhat less homozygous animals. All of the mice were two to five months old at the beginning of the experiments; the average strain weights were: C57BR/cd, 23 grams; AKR, 29 grams; DBA/1, 20 grams; F_1 of C57BR/cd♀ x AKR♂, 25 grams; F_1 of AKR♀ x C57BR/cd♂, 20 grams; and albino Swiss, 30 grams. Mice were kept in plastic cages partially filled with sawdust and the cages were washed weekly. The cages, with five to 10 mice apiece, were stored in a constant

temperature (22°C), air-conditioned room and were given mouse food (Purina) and water ad libitum.

Sera: Mice were lightly etherized and blood samples were obtained from the cavernous venous sinus through the infra-orbital route with the aid of a glass Pasteur pipette. With practice, 0.3 to 0.75 ml. of blood could be removed at weekly intervals from each mouse without significant morbidity or mortality. Mice were recaged after recovery from anesthesia. All mice were bled on each scheduled day. Mortality averaged under two per cent per bleeding.

After clotting at room temperature, the blood samples were centrifuged twice at 2000 to 2500 RPM. The separated sera were inactivated at 56°C for 30 minutes and subsequently frozen at minus 28°C until the analyses.

Antigens: Two strains of Type A influenza virus were employed as antigens, these were the PR-8 and A/Japan 305/57 strains. These viruses were maintained by serial allantoic egg passage, they were not mouse adapted. Nine to 10 day embryonated eggs were inoculated with 0.1 ml. of a 10^{-3} to 10^{-5} dilution of the previous passage. After incubation at 37°C for 42 hours the eggs were refrigerated and the allantoic fluid harvested by syringe using a needle with multiple perforations. After every third egg passage each of the viruses was tested for contamination or gross change by cross hemagglutination-inhibition tests with standard antisera originally obtained

from the Public Health Service Communicable Disease Center (Montgomery, Alabama). There was not evidence of contamination or change during the seven to eight egg passages made for each virus during the one year course of these experiments.

Bovine serum albumin (BSA) was used as the antigen in several of the experiments. BSA, Armour Fraction V, was dissolved in isotonic phosphate buffer and sterilized by filtration through sintered glass. It was diluted to the desired concentration with the same diluent. Alum precipitated BSA was prepared by the addition of seven per cent aluminum chloride slowly to a 10 mg. per ml. BSA preparation to the point of maximal turbidity. This preparation, which contained more than two-thirds of the total protein in the precipitate was then brought to pH 7 by the addition of N/10 NaOH.

Purification of the Viruses: The virus-containing allantoic fluids were first clarified by centrifugation for 10 minutes at 0°C at 10,000 RPM in a PR-2 centrifuge followed by ultracentrifugation for 90 minutes at 0°C at 30,000 RPM in a Spinco centrifuge. Four-fifths of the supernates containing insignificant hemagglutinin activity were discarded. The pellets were carefully resuspended in pH 7 phosphate buffer to approximately the original volumes. The resulting suspensions were centrifuged once again for 90 minutes at 0°C at 30,000 RPM and the pellets resuspended to about one-half the original volumes. Five hundred units of penicillin and 500 micrograms

of streptomycin were added per ml. and the preparations stored in aliquots at 0-4°C until use.

Vaccines: The purified virus preparations were inactivated with betapropiolactone (BPL), a new non-toxic rapidly acting virucidal agent active in very low concentrations and said to inactivate viruses without loss of antigenicity (39, 40, 41). Equal volumes of purified virus and of 0.05% BPL in distilled water were mixed at 0°C to give a concentration of 0.025% BPL. The solutions were incubated at 37°C for two hours to permit hydrolysis of the BPL and immediately refrigerated at 0-4°C until use. The vaccines were not toxic or infective for eggs in the undiluted state.

Egg infectivity studies: The purified virus preparations before and after inactivation with BPL were tested for egg infectivity. Eggs were inoculated with 10-fold dilutions of the virus preparations, four to six eggs per dilution, as described under Antigens above. Allantoic fluid from individual eggs was tested for hemagglutination of chicken erythrocytes and the 50 per cent infective doses (EID_{50}) were computed according to the method of Reed and Muench (42).

Virus for hemagglutination-inhibition (HI) tests: HI test antigen was purified virus. Viral titrations of the preparations were made by hemagglutination (HA) tests using viral dilutions (0.3 ml.) and a 0.5% suspension of chicken eryth-

rocytes (0.3 ml.). The endpoint was 50 per cent or 2 (+) hemagglutination. A dilution of each preparation containing three HA doses was selected as the HI antigen, i.e., a dilution which was three times as concentrated as that giving the HA endpoint.

Virus for complement-fixation (CF) tests: CF test antigen was allantoic fluid clarified by low speed centrifugation. Optimal antigen concentrations for CF were determined by "checkerboard" titrations using allantoic fluid dilutions (0.5 ml.), mouse immune serum (0.2 ml.), five $C'H_{50}$ units of guinea pig complement (0.5 ml.), and sensitized sheep erythrocytes (0.3 ml.). That dilution of each virus which gave maximum fixation of complement at the highest dilution of serum subsequently was used as the respective CF antigen.

Chicken antisera: A chicken antiserum was prepared for each virus to be employed as a standard serum to be retitrated with each HI analysis as a check on reproducibility of the analyses. Chickens were given 10 ml. intravenously and 20 ml. intraperitoneally of each purified virus preparation on the first day followed by 20 ml. by each route on the fourth day. One week following this second injection they were exsanguinated and the sera separated and frozen. The PR-8 antiserum HI titer was 3000, it did not cross react with the Japan 305/57 virus. The titer of the Japan 305/57 antiserum

was 300, it cross reacted to less than a 1:10 dilution with the PR-8 virus.

Complement (C'): Commercially prepared complement from Carworth Farms consisting of a fresh frozen pool of guinea pig serum was used. The C' was titrated by the sheep cell hemolytic system and found to contain greater than 200 $C'H_{50}$ units per ml. It was diluted for the analyses so that 0.5 ml. contained five $C'H_{50}$ units.

Chicken erythrocytes for the HI tests: A suspension of chicken red cells was prepared by washing the cells three times with NaCl followed by suspension in saline to yield an approximate 0.5 per cent suspension. No red cells stored for more than four days post-bleeding were used.

Sheep erythrocytes for the CF tests: One volume of sheep blood was collected into two volumes of Alsever's solution and stored in this form for no less than one day and no more than 30 days before use. Before being used, the cells were washed once with NaCl, thrice with Veronal buffer (VB) and suspended in VB. They were standardized on the Beckman spectrophotometer to an optical density of 0.680. A complete description of the technique of sensitization may be found in Osler, Strauss and Mayer (43). In brief, the standardized erythrocytes were sensitized with an equal volume of amboceptor (serum of rabbits immunized

with boiled sheep erythrocyte stromata) estimated to give about 1000 antibody molecules per red cell. The resulting suspension was incubated at room temperature for 30 minutes and then refrigerated until use.

Diluents: Isotonic sodium chloride (NaCl) was constituted as a 0.85 per cent solution.

Phsophate buffer, pH 7 was made by the addition of seven parts of 0.10 M Na_2HPO_4 (1.42%) to two parts of 0.15 M KH_2PO_4 (2.04%). The pH was checked by meter.

Alsever's anticoagulant solution consisted of 2.05 per cent glucose, 0.8 per cent trisodium citrate, and 0.42 per cent NaCl in distilled water. The pH was adjusted to 6.1 with five per cent citric acid.

Veronal buffer (VB) was made up as a five times isotonic stock solution which consisted of 4.19 per cent NaCl, 0.126 per cent $NaHCO_3$, 0.15 per cent sodium barbital and 0.23 per cent barbital in distilled water. Prior to use the stock was diluted to isotonicity with distilled water containing five ml. of 0.216 M $MgCl_2 \cdot 6H_2O$ and five ml. of 0.03 M $CaCl_2 \cdot H_2O$ per liter.

Gelatin veronal buffer (Gel VB) was made as VB and contained in addition 50 ml. of one per cent gelatin per liter.

The hemagglutination-inhibition (HI) tests: Two-fold serial serum dilutions were made in VB from the heated mouse serum

beginning with a 1:10 to 1:30 dilution. The tests were run in Konte hemagglutination tubes. The reaction mixtures consisted of 0.3 ml. of mouse serum dilutions, 0.3 ml. of a dilution in VB of purified virus containing three HA doses, and 0.3 ml. of a 0.5% suspension of chicken erythrocytes in NaCl. The tubes were allowed to stand for 20 minutes at room temperature before the erythrocytes were added. Virus and red cells were added with the aid of an automatic pipette. After 40-60 minutes at room temperature the patterns of the settled erythrocytes were read visually taking 2 (+) hemagglutination as the endpoint. The reciprocal of the serum dilution producing this reactivity was recorded as the titer. Each analysis contained these controls: a tube without virus at the lowest serum dilution as a test for spontaneous hemagglutination; tubes without virus or serum; a titration of the standard chicken antiserum to detect variance between analyses; and, a hemagglutination titration of the virus in the absence of serum. With low-titered sera of certain strains (C57BR/cd, DBA/1 and both F_1 strains) there were hemagglutinins for chicken erythrocytes in the sera. These sera were absorbed twice with chicken erythrocytes in packed form in a 1:10 serum dilution at $0^{o}C$, which was always sufficient to remove the agglutinins. In no case was a serum absorbed with chicken erythrocytes used in a CF analysis.

The complement-fixation (CF) tests: Two-fold serial serum

dilutions were made from mouse serum in VB beginning with a 1:10 to 1:30 dilution; the serial dilutions used in this test were in most cases the same ones as used in the HI tests. The tests were performed in 13 x 100 mm. tubes. After addition of the serum dilution in 0.2 ml. volume, the tubes were cooled to 0°C and 0.5 ml. of C' (five $C'H_{50}$ units) was added to each tube. Then 0.5 ml. of the optimal dilution of allantoic fluid containing virus was added to each tube except the appropriate controls and all tubes were placed at 0°C overnight followed by a 20 minute incubation at 37°C. Subsequently 0.3 ml. of sensitized sheep erythrocytes in Gel VB was added to each tube as the indicator for residual C' and the tubes were incubated further for 60 minutes at 37°C with intermittent shaking. Complement, virus and sensitized erythrocytes were added with an automatic pipette. The tubes were then centrifuged and the degree of lysis determined visually taking 2 (+) (50 per cent) lysis as the endpoint for expression of titer. The reciprocal of the serum dilution producing this reactivity was recorded as the titer. Each analysis contained these controls: a tube without virus at the lowest serum dilution as a test for anti-complementary action of the serum; a control without serum as a test for anti-complementary action of the allantoic fluid virus preparation; a titration of a mouse serum repeated with each analysis to detect variance; and a complement titration containing only varying dilutions of complement plus diluent

and sensitized erythrocytes. Rarely the serum was anti-complementary at the 1:10 dilution, and in these cases this dilution was discarded. In no case was either a 1:20 serum dilution or the virus preparation anti-complementary.

The CF test performed in the BSA experiments was identical in volumes and procedure with that just described except where otherwise indicated in the text.

Capillary ring precipitin test: This test was used only in the BSA experiments. First antigen, then serum was drawn by capillary action into Kimble melting-point tubes, the tubes being wiped before insertion into the serum. They were placed vertically in plasticene and read at two and 24 hours. The precipitate was graded as zero to four (+).

PROCEDURES

Part I: The immune response of four inbred strains of mice

Part I was a study of the immune response of four strains of mice to two related Type A influenza virus vaccines. The strains of mice used and their ages at the beginning of the experiment were: 1) C57BR/cd, three months; 2) DBA/1, four months; 3) AKR, four months; and 4) albino Swiss, three months. Only females were employed.

The PR-8 purified virus before inactivation had an EID_{50} of $10^{8.3}$ per ml. and an HA titer of 400. Inactivation with BPL involved a 1:2 dilution of this preparation and made the virus non-infective for eggs. All mice received 0.5 ml. of this vaccine intraperitoneally on each of the scheduled days. There is no simple method of comparing doses of inactivated virus particles, therefore, in order to obtain a rough idea of comparative dosage between the vaccines of Part I and Part II the vaccines were compared in terms of their EID_{50}, although in reality they were not infective for eggs. Thus, for PR-8, the dose of vaccine (0.5 ml.) was a 1:2 dilution of the original purified preparation and thus contained one-fourth the original quantity of virus per ml. The "equivalent EID_{50}" was therefore 0.5×10^{8}.

The Japan 305/57 virus preparation before inactivation had an EID_{50} of $10^{5.6}$ per ml. and an HA titer of 100. Inactivation likewise involved a 1:2 dilution and the mice

similarly received 0.5 ml. of the resulting vaccine. The "equivalent EID_{50}" was 1×10^5.

Identical numbers of mice received each vaccine. Fifteen mice from each of the four strains received PR-8 vaccine and 15 received Japan 305/57 vaccine. The 15 mice from each strain were in turn arbitrarily sub-divided into three groups of five mice apiece, and each of these three groups was treated as a unit, caged separately and their sera pooled. All 60 mice receiving each vaccine were treated identically as regards inoculation and bleeding. This protocol of division of the mice of each strain into sub-groups was followed in order to furnish sufficient serum for both serologic tests and to give also gross information about variance within each strain. All of the serum samples obtained were analyzed by both the HI and CF tests.

The experiment extended over a period of six weeks. The times of injection and bleeding given below were identical for mice receiving either vaccine and the volume of the vaccines was the same in each injection. The mice received a total of five intraperitoneal injections spaced over the first three weeks and were bled a total of eight times. Every mouse was inoculated and bled on the scheduled days.

Day	0	mice bled
	1	mice injected
	4	mice bled
	5	mice injected
	7	mice bled

(continued)

Day 8	mice injected
14	mice bled
15	mice injected
21	mice bled
22	mice injected
28	mice bled
35	mice bled
42	mice bled

The mice remained healthy and active throughout the experiment. Mortality, except for three mice which died intercurrently, was confined to the time of bleeding and was probably due to anesthesia or exsanguination. The numbers given below represent the number of animals of the original 15 in each strain remaining alive after 42 days.

PR-8	C57BR/cd	11	Japan 305/57	C57BR/cd	13
	DBA/1	8		DBA/1	10
	AKR	12		AKR	12
	Swiss	10		Swiss	11

Cumulative mortality was thus 32 per cent for the PR-8 experimental mice and 24 per cent for the Japan 305/57 experimental mice.

Part II: The heritability of the immune response

Part II was a study of the characteristics of the immune response of two of the strains studied in Part I and of the first filial generation (F_1) of the reciprocal matings of these two strains. Unfortunately, due to limitations of time, the F_2 could not also be studied. The strains of mice used and their ages at the beginning of the experiment were: 1) C57BR/cd, five months; 2) AKR, five months;

3) F_1 of C57BR/cd♀ x AKR♂, two to three months; and 4) F_1 of AKR♀ x C57BR/cd♂, two to three months. Only females of the first two strains listed were employed while mixed sexes of the F_1 progeny were used. The F_1 strains were bred in this laboratory from parental strains obtained from the Jackson Laboratory. Part II took place one year after the study of Part I.

The PR-8 and Japan 305/57 viruses represented further egg passages of the viruses used in Part I. They were purified and inactivated as described earlier. In an attempt to obtain the same dosage for each virus as used in Part I, the inactivated preparations were given to the animals in a dosage which was of roughly the same "equivalent EID_{50}" as the respective viruses of Part I. It must be remembered however, that the vaccines were in reality not infective for eggs. For PR-8 the EID_{50} of the purified virus preparation was $10^{8.6}$ per ml. and the HA titer was 480. Inactivation involved a 1:2 dilution. It was calculated that about 0.25 ml. of the newly prepared vaccine had an "equivalent EID_{50}" equal to that of 0.5 ml. of the PR-8 vaccine of Part I (0.5×10^8). Therefore 0.25 ml. was the dose of PR-8 vaccine given intraperitoneally in each scheduled inoculation.

Similarly, the Japan 305/57 purified virus had an EID_{50} before inactivation of $10^{5.2}$ and an HA titer of 600. Inactivation reduced the concentration by one-half and

calculations showed that 1.25 ml. of this vaccine had the same "equivalent EID_{50}" as 0.5 ml. of the vaccine of Part I, namely 1 x 10^5. Thus 1.25 ml. was the dose of Japan 305/57 vaccine given intraperitoneally on each scheduled day.

Due to improvements in technique and the desirability of being able to detect possible inheritance patterns, the serum of each mouse in Part II was analyzed individually. Organization of this part of the experiment with respect to caging, numbers of animals and general protocol was the same for mice receiving either PR-8 virus vaccine or Japan 305/57 virus vaccine. Identical numbers of mice received each vaccine. Six mice from each of the four strains received each virus vaccine. The experiment extended over a period of 28 days. The schedule was the same for the mice receiving either vaccine and the volume of each preparation given was the same in each injection. The mice received a total of four intraperitoneal injections spaced over the first two weeks and they were bled a total of six times. All mice were injected and bled on the scheduled days, there was no serum pooling.

Day	0	mice bled
	1	mice injected
	4	mice bled
	5	mice injected
	7	mice bled
	8	mice injected
	14	mice bled
	15	mice injected
	21	mice bled
	28	mice bled

Data on mortality is given below. The mice remained healthy, deaths usually occurred on the day of bleeding during or immediately following the procedure. Three mice died intercurrently. The numbers of mice given below are the number of the original six per strain remaining alive after 28 days.

PR-8	C57BR/cd	5	Japan 305/57	C57BR/cd	5
	AKR	4		AKR	4
	F_1: C57BR/cd♀ x AKR♂	4		F_1: C57BR/cd♀ x AKR♂	3
	F_1: AKR♀ x C57BR/cd♂	3		F_1: AKR♀ x C57BR/cd♂	5

Cumulative mortality was thus 33 per cent for the PR-8 experimental mice and 29 per cent for the Japan 305/57 experimental mice.

Part III: The response of inbred mice to BSA

Part III was intended as a study of the immune response of four strains of mice to a partially purified protein antigen. The antigen chosen was bovine serum albumin (BSA). The strains of mice employed and their ages were the same as those employed in Part I. Sera were analyzed by multiple serologic tests as described under Results.

A pilot study using BSA as antigen was initiated in 10 mice from each of the four strains. All mice were bled and injected on the scheduled days. The BSA, either soluble or alum-precipitated, was given intraperitoneally (ip) or subcutaneously (sc) in the listed dosages. The experiment extended over 48 days; the mice received five injections and

were bled a total of six times.

Day	0	mice bled
	1	mice given 1 mg. soluble BSA ip
	4	mice given 1 mg. soluble BSA ip
	7	mice bled
	8	mice given 1.5 mg. alum-BSA ip
	14	mice bled
	15	mice given 1.5 mg. alum-BSA sc
	21	mice bled
	22	mice given 1.5 mg. alum-BSA ip
	33	mice bled
	48	mice bled

The mice thus received a total of 6.5 mg. of BSA apiece.

As the above immunization schedule failed to reveal antibody, a further study using protocols of intensive antigenic stimulation was initiated. Only Swiss mice were used in this portion of the experiment. Two separate immunization schedules involving heavy stimulation were devised, labelled as Groups I and II below, respectively. There were 10 mice involved in each of the groups; all mice were bled and injected on the respective scheduled days. This experiment extended over a period of 39 days. The schedules for Groups I and II are given below:

Day	0	mice bled
	1	mice given 1.14 mg. soluble BSA iv and 2 mg. alum-BSA ip
	2	mice given 1.14 mg. soluble BSA iv and 2 mg. alum-BSA ip
	3	mice given 1.14 mg. soluble BSA iv and 2 mg. alum-BSA ip
	4	mice given 3 mg. soluble BSA iv and 3 mg. alum-BSA ip
	5	mice given 3 mg. soluble BSA iv and 3 mg. alum-BSA ip
	6	mice given 3.5 mg. soluble BSA iv and 4.5 mg. alum-BSA ip
	7	mice given 3.5 mg. soluble BSA iv and 4.5 mg. alum-BSA ip
	14	mice bled
	15	mice given 3.5 mg. soluble BSA iv and 4.5 mg. alum-BSA ip
	16	mice given 5 mg. soluble BSA iv and 6.45 mg. alum-BSA ip
	17	mice given 5 mg. soluble BSA iv and 6.45 mg. alum-BSA ip
	18	mice given 5 mg. soluble BSA iv and 9.7 mg. alum-BSA ip
	19	mice given 5 mg. soluble BSA iv and 12.9 mg. alum-BSA ip
	20	mice given 5 mg. soluble BSA iv and 12.9 mg. alum-BSA ip
	21	mice given 5 mg. soluble BSA iv and 12.9 mg. alum-BSA ip
	32	mice bled
	39	mice bled and tissues taken for analysis.

Group II

Day 0 mice bled
1 mice given 3 mg. alum-BSA ip
2 mice given 3 mg. alum-BSA ip
3 mice given 3 mg. alum-BSA ip
4 mice given 1.5 mg. soluble BSA iv and 3 mg. alum-BSA ip
5 dosage and schedule from day 5 through day 39 is identical with Group I.

The total dose per mouse in Group I was 49.64 mg. BSA intravenously and 80.15 mg. intraperitoneally for a total of 129.79 mg. For Group II the intravenous dosage was 45 mg. and the intraperitoneal dosage 83.15 mg., for a total of 128.15 mg. per mouse.

EXPERIMENTAL RESULTS

In the tables column "A" gives the titers obtained in the serological analyses and represents the titer of serum pools in Part I and the titer of individual mice in Part II. Column "B" gives the mean geometrical titer of the titers in column "A" and it is this figure, representative of the titer of each strain for each day of the study which was used in graphing the results.

The "controls" in the tables and figures were sera which were retitrated with each analysis to determine reproducibility of the tests. In the HI tests this was a chicken antiserum to each virus while in the CF tests it was a mouse antiserum to each virus.

Part I: The Immune Response of Four Inbred Strains of Mice

General Results (Tables 1-4, Figures 1-4) It may be seen from the tables and graphs that the mouse strains demonstrated variation of strain titer with time which was of a greater magnitude than could be accounted for by lack of reproducibility of the serological tests. It was unfortunately not possible to analyze the results of Part I statistically since the use of serum pooling precluded intra-strain variability in titer, and there is no statistical technique to compare series of identical numbers.

The pre-inoculation sera were in all cases negative for antibody by both the HI and CF tests. Neither PR-8 nor Japan 305/57 antisera cross-reacted with the heterologous virus in either serological test.

Antibody was usually first demonstrable after two or three injections of antigen, on day seven or day 14, and from this point in each case the antibody response curve continued a steep rise to maximal titer. On day 14 or day 21 the slopes of the curves usually decreased and tended to flatten out to a more gradual increase in titer or to a plateau. Continued antigenic stimulation during this period of one to three weeks of approximately maximum response served to maintain but not to increase markedly the titer. This gradual increase in titer or plateau persisted for one week or at most two weeks following the cessation of antigen inoculations and was immediately followed by a sharp fall in titer, generally in an exponential manner. In several instances it was possible to calculate the antibody half-life from this portion of the curve.

This general pattern of response held true in general for both viruses and for both antibodies to each virus but there were, however, some differences between the viruses and their antibodies within this common framework. The PR-8 virus engendered higher titers in both the HI and CF tests than did the Japan 305/57 virus; the results of Part II indicated that this was not entirely due to the great differential in dosage of the two viruses received by the animals.

The PR-8 animals tended to show an earlier appearance of antibody with an earlier attainment of maximum titer as well as a longer persistence of antibody after the cessation of antigenic stimulation. The PR-8 curves therefore were generally higher and wider with an early rise to maximum titer and a long period at or near this level. The CF antibody appeared later than did the HI antibody and had lower titers, likewise, the CF antibody had a shorter half-life than the HI antibody, a confirmation of the well known characteristics of these two antibodies.

The four mouse strains tended to group themselves into two differently reacting pairs: the two brown strains, C57BR/cd and DBA/1 comprising one pair and the two albino strains, AKR and Swiss the other pair. This pattern usually was most marked during the early parts of the antibody response curves, that is, during the rise to the peak titer attained although it was sometimes true also for the plateau and exponential decline of the antibody level. The C57BR/cd and DBA/1 strains reached higher titers than the other two strains in the HI test while this relationship was reversed in the results of the CF test. Similarly, the two brown strains had shorter antibody half-lives in the CF results than did the albino strains, half-life information from the HI results was incomplete. The Swiss strain, although paired with the AKR strain during the first part of the curves thereafter tended to react differently from the other strains in all but the Japan 305/57 CF analysis.

PR-8 Virus, HI response (Table 1, Figure 1) The results of this study demonstrated the grouping of the four strains into two differently reacting pairs during the first half of the experiment followed by the loss of this pattern during the remainder of the study, the strains then varying independently. The AKR and Swiss strains tended to react to lower titers than the DBA/1 and C57BR/cd strain and their maximal titers were about two-thirds those of the latter strains. Throughout the experiment the albino animals reacted very similarly and their antibody response curves lay below those of the brown strains to the point of antibody decline although the difference was not striking after the twenty-first day. All strains had demonstrable antibody by the fourth day after only one injection of virus, this early appearance of antibody was evident elsewhere only in the PR-8 HI study of Part II. The AKR and Swiss strains had an apparent drop in titer between the fourth and seventh days which was followed by a rise in titer. This drop in titer probably represented an anomaly of the experimental design due to the fact that the day four bleeding was followed on day five by an injection of vaccine. It is likely, therefore, that antigen was circulating in the serum on day seven, the time of the next serum sample, and was responsible for a falsely low level due to the neutralization of the antibody in the serum. The two brown strains reached their maximum titer on

day 14 after a total of three injections of antigen while the albino strains reached maximum titer on day 21, after four injections of antigen. This time difference may not have been that great due to the absence of intermediate serum samples. After reaching maximal titer on day 14 or 21 all four strains remained at about the same level of response through day 35 and continued antigenic stimulation during this period, on days 15 and 22, did not serve to change the titers markedly. After day 35 all of the strains except the Swiss began to decline sharply to reach approximately one-half their respective maximum titers by day 42.

Japan 305/57 Virus, HI response (Table 2, Figure 2) The results of this experiment illustrated also the pairing of the strains which was here, however, sharply limited to the first three weeks. During this part of the curves the AKR and Swiss again tended to react to lower titers. It will be noted here that the curves were still rising at the end of the antigen inoculations and no true plateau was evident although there was a change in slope on day 14 for the brown strains and on day 21 for the albino strains. The maximal titer which was reached in this experiment was lower for the AKR and Swiss strains than it was for the C57BR/cd and DBA/1 strains and in addition the albino strains demonstrated a later appearance of antibody. The dosage of antigen received by the Japan 305/57 animals in terms of either "equivalent EID_{50}"

or HA doses was much less than received by the PR-8 animals, and, if a similar reactivity of the mouse strains to the two viruses is assumed, the later appearance of antibody, the lack of a plateau and the fact that the curves did not reach peak production of antibody could be explained on the basis of the differential in dosage. Notable in this HI experiment also was the pattern of the Swiss strain which promptly began to decline exponentially after day 21 despite continued injections of antigen. After day 28, one week following the last injection of vaccine, the C57BR/cd, DBA/1 and AKR strains began to decline sharply in titer. In the case of the C57BR/cd and AKR strains where there were three weekly titer determinations in a straight exponential decline, the Japan 305/57 HI antibody half-life could be computed. For C57BR/cd this was 4.4 days and for AKR it was 6.9 days. The half-life was not computed for the Swiss strain since its antibody decline began while antigen was still being given.

PR-8 Virus, CF Response (Table 3, Figure 3) Some of the characteristics of the HI response were also present in this CF study however the patterns here were not very clear. Only the albino strains, AKR and Swiss were convincingly paired in their response and then only to the peak titer reached. This was achieved on day 28 after five injections of antigen. The C57BR/cd and DBA/1 strains were not paired. Strain DBA showed a definite plateau between days 14 and 28 despite

two further antigen inoculations during this period. The C57BR/cd strain dropped in titer between days 14 and 21 only to rise to its original level of day 14 on day 28, this degree of variability might have been accounted for by variations in the reproducibility of the test and C57BR/cd therefore may have had a plateau during this time period. It appears that the AKR and Swiss strains had not reached their maximum titer as the curves were still rising when antigenic stimulation was stopped. Here also, it will be noted that the lag period was about one week following the last inoculation of antigen before the titers began to decline markedly. Since both the AKR and Swiss strains had three points on a straight decline their CF antibody half-lives could be determined. For AKR this was 4.9 days and for Swiss it was 11.9 days.

Japan 305/57 Virus, CF Response (Table 4, Figure 4) The results of this study demonstrated most conclusively the patterns observed in Part I. The pairing of the strains was here very evident and it extended beyond the period of rise to maximum titer and included likewise the decline of the antibody level. After an initial steep rise in titer of both pairs of animal strains the slope of the curves diminished on day 14 and continued to rise slightly. In this study, in contrast to the HI responses, the two albino strains had higher titers throughout the entire antibody response. It

was notable that throughout their courses the two strains of each pair reacted to virtually identical titers. Maximum titer was attained for both pairs on day 28 and since all of the curves were still rising, it is probable that the strains had not reached their maximum response. Maximal titers of the two brown strains were about one-half those of the albino strains. One week after the last antigen injection all four strains began an exponential decline in titer. Half-lives of the CF antibody could be computed for each strain and were: AKR, 4.4 days; Swiss, 4.5 days; C57BR/cd, 2.9 days; and DBA/1, 2.9 days.

Part II: The Heritability of the Immune Response

General Results (Tables 5-8, Figures 5-8) The results of Part II were consistent and showed a similar type of reactivity which was independent of the virus or antibody test involved. Therefore it is possible to give the findings in general terms.

Once again the graphs and tables demonstrated antigenic effect as seen by a comparison of the strain curves with the controls, i.e., there was a rise in antibody production which could not be accounted for by lack of reproducibility of the tests or by a random distribution of reactivity. Statistical analysis was done on the results of Part II and is given at the end of this section.

Pre-inoculation sera were always negative for antibody.

Neither PR-8 nor Japan 305/57 sera cross-reacted with the heterologous virus in either antibody test. Study of the individual mouse titers of the F_1 strains given in the tables revealed intra-strain variation but no consistent subgroupings, rather the individual titers appeared to conform rather closely to the theoretical distribution of normal probability. The mean geometrical titer was therefore considered representative of individual titers for the purpose of graphing the results.

The attempt to give the same dosage of each virus in Parts I and II was unsuccessful for the dosage of virus in Part II appears to have been greater than that of Part I, at least insofar as the dosage is reflected in the serological test results. There were several observations which pointed to a greater dosage in this part of the experiment: 1) there was an earlier appearance of antibody (in all cases by day seven), 2) the early rise to maximum titer was very steep, 3) maximum titer was achieved earlier in Part II and 4) all of the experiments demonstrated a "plateau effect" at maximum titer. The actual value of the maximum titer reached was not considered a function of dosage; this point is further considered in the Discussion.

At day 14 in these experiments the slopes of the curves of antibody production changed, a confirmation of the results of Part I, however in these experiments this was almost always a change to a plateau of titer with time. This plateau of

maximum titer extended from the fourteenth through the twenty-first day. A further dose of vaccine was given in all cases on day 15, however it is obvious that the resulting titer on day 21 was little changed from that present on day 14. This is a confirmation once again of the fact that each virus and each antibody has a maximal attainable titer which is independent of the number of times a given dose was given and thereby, presumably, of the total dose of antigen. One week following the cessation of the antigenic stimulation the antibody curves began to decline. Some of the strains appeared to have exponentially declining titers, but since the animals were not followed for longer than one week after the beginning of the loss of antibody titer, it was not possible to ascertain this probability.

Within the general pattern of reactivity there was internal variation for both viruses and both antibodies studied. Animals which received PR-8 virus vaccine had higher titers in the HI test than those which received Japan 305/57 vaccine, while the CF titers were identical in the antisera to either virus. For each virus the respective CF titers were lower than the HI titers throughout the respective curves of antibody response.

Once more, and here very dramatically, the four mouse strains of Part II tended to react according to two different patterns. The AKR and both F_1 strains reacted very similarly if not identically up to the time of incipient antibody decline

while the C57BR/cd strain manifested a different pattern of reactivity during this period of time. It will be remembered that in Part I the AKR and C57BR/cd strains were members of the two differently reacting mouse strain pairs respectively. This grouping of antibody response of the mouse strains was manifest in all of the antibody titrations of Part II, it was paralleled in extent only by the Japan 305/57 CF study of Part I.

More specifically, it may be seen that the C57BR/cd strain always reacted to lower titers in both serological tests than did the other three strains. In addition the curve of this strain had a distinctive shape in that on day 14, when the slope of the curves of the other strains assumed a horizontal position, the C57BR/cd strain curve showed only a reduction in slope and continued to rise somewhat until one week following the last antigen inoculation. The response of the C57BR/cd strain in the PR-8 HI study was somewhat anomalous during this first portion of the curve and its unusual pattern there cannot be explained. The maximum titer reached by the C57BR/cd strain was always between one-third and one-half that of the remaining strains, this held true regardless of the virus or antibody test involved, it was likewise observed in the Japan 305/57 CF experiment of Part I.

If actual titers are ignored, the similarity of all of the graphs of Part II is very striking. This observation has implications for a consideration of the antibody response

to two closely related antigens and to antigenic components of the complex, multiple-antigen-containing viral particle.

Statistical analysis A rank sum test (44) was used to analyze the results. The individual titers of the animals in each strain were compared with those of every other strain for each day of the study. The results of the analyses for the antibodies studied is given below.

PR-8, HI The C57BR/cd strain was statistically different at at least $P = 0.05$ and usually at $P < 0.01$ from all other strains on days 7, 14, 21 and 28. There were no statistical differences between the AKR and F_1 strains on days 7, 14, 21 or 28.

PR-8, CF The C57BR/cd strain was statistically different at at least $P = 0.05$ and usually at $P < 0.01$ from the AKR strain on days 14, 21 and 28; from the F_1 of C57BR/cd ♀ x AKR ♂ on days 7, 14, 21 and 28; and from the F_1 of AKR ♀ x C57BR/cd ♂ on days 7 and 28. There were no statistical differences between the AKR and F_1 strains on days 7, 14, 21, or 28.

Japan 305/57, HI The C57BR/cd strain was statistically different at at least $P = 0.05$ and usually at $P < 0.01$ from the AKR strain on days 7, 14, and 28; from the F_1 of AKR ♀ x C57BR/cd ♂ and the F_1 of C57BR/cd ♀ x AKR ♂ on days 7, 14, 21, and 28. On day 28 AKR and both F_1 strains were significantly different from one another at $P = 0.05$. There were no other differences.

Japan 305/57, CF The C57BR/cd strain was statistically different at at least P = 0.05 from AKR on days 14, 21 and 28; from the F_1 of AKR ♀ x C57BR/cd ♂ on days 14, 21 and 28; and from the F_1 of C57BR/cd ♀ x AKR ♂ on days 14 and 28. On day 7 F_1 of AKR ♀ x C57BR/cd ♂ were statistically different at P = 0.05 from all other strains; on day 28 AKR was different at P = 0.05 from F_1 of AKR ♀ x C57BR/cd ♂. There were no other differences.

"P" must be interpreted in the statistical sense and is defined as the probability of obtaining a difference as large or larger than that observed if in fact the strains did not differ at all. A "P" value of 0.05 signifies a probability of 1 in 20, a "P" of 0.01 a probability of 1 in 100. In addition it must be mentioned that the strains were compared day by day and that the analysis does not take into account the difference of one entire curve from another. Interpretation of the results of the statistical analysis in this light further emphasizes the similarity of the AKR and F_1 strains to one another and their difference from the C57BR/cd strain.

Part III: The Response of Inbred Mice to BSA

Mouse sera obtained from the six bleedings of the first immunization schedule for BSA described in Procedures were analyzed by CF using as antigen a BSA dilution which was

optimal in a "checkerboard" analysis with a BSA rabbit anti-BSA system. Five units of C' were used, the volumes of serum, antigen, C' and sensitized sheep erythrocytes as well as the conditions of testing were exactly as used in analyzing the anti-viral sera. All of the serum samples proved to be negative for antibody. The sera were tested in the ring precipitin test in capillary tubes with a number of concentrations of BSA, there was no detectable precipitation. The possibility arose that perhaps the mouse BSA system had a markedly different optimum combining ratio from the rabbit system from which the optimum antigen dilution was derived. If this situation prevailed, it might have been possible that antibody was missed due to the presence of antigen or antibody excess. A "checkerboard" analysis using day 21 sera from the immunized mice was run with varying dilutions of the sera and of BSA by the CF technique. There was no detectable amount of C' fixed. Then, proceeding on the possibility that the mouse albumin system might be analogous to the horse anti-pneumococcal system which does not fix C' in the usual CF protocol but does, in the presence of a large excess of C', fix some complement which may then be tested for by titration of the remaining C'. An assay of this type employing 50 units of guinea pig C', several dilutions of the mouse day 33 sera and two concentrations of BSA was performed. Titration for residual C' failed to show any fixation of C' beyond that

amount remaining in the controls which contained neither antigen nor antibody.

Since mice are less efficient than many other laboratory animals in producing antibody, more intensive schedules of immunization were devised on the chance that the mice had received insufficient antigen to stimulate the production of antibodies. Group I was a schedule of heavy stimulation with BSA given intravenously and intraperitoneally, Group II was a similar schedule which began with lighter stimulation to rule out the possibility of immune paralysis as described by Felton (45). In the schedules of Groups I and II there was an eight day rest period between days seven and 15 during which antigen was not given. Upon resumption of antigen inoculations on day 15 the mice in both groups developed anaphylactic shock and about 30% died. This confirmed work which had shown that BSA could produce anaphylactic shock in mice (46, 47, 48) and incidentally demonstrated that the mice were not suffering from immune paralysis. It likewise established the existence of some antibody in the mice to account for the observed shock upon reinjection of BSA.

In none of the sera from the three experimental bleedings of either the Group I or Group II mice was antibody demonstrable by the conventional CF test with either two or five units of complement. The capillary tube precipitin test gave similar results. "Checkerboard" analyses with varying dilutions of

antigen and serum in both the CF and ring precipitin tests likewise failed to demonstrate antibody. Analysis for precipitating antibody using Ouchterlony's technique of precipitation in agar (49) was negative as were analyses employing Preer's reportedly more sensitive technique of precipitation in semi-liquid agar in fine glass tubing (50). On the possibility that mouse C' components attach in an unusual manner, CF analysis was performed without the usual overnight period at $0^{\circ}C$, this was also unsuccessful. Immune adherence analyses (51) and hemagglutination with tannic acid treated erythrocytes (52, 53) were inconclusive.

A test was then devised to detect any possible non-precipitating and non-complement-fixing antibody circulating in the mouse serum. This test was based on the assumption that were such an antibody present, it would combine with BSA thus decreasing the amount of uncombined BSA present. This reduction of the BSA concentration should then be detectable in the presence of an adequate indicator system. A standardized BSA rabbit anti-BSA system was selected as indicator. Accordingly a titration of a rabbit anti-BSA serum with an optimal dilution of BSA for the system was set up in capillary tubes. This same dilution of BSA was also incubated with varying dilutions of the day 33 mouse sera in test tubes. After incubation these BSA-mouse serum mixtures were used as the antigens in retitrations of the rabbit anti-BSA serum

in capillary tubes. After allowing for the reduction in BSA concentration as a result of dilution with mouse serum the resulting titration of the rabbit serum was the same before and after incubation with the mouse serum. This indicated that the concentration of BSA had not changed. The test gave good evidence that the mice had no circulating antibody to BSA.

The logical deduction was that the mice had antibody to BSA in their tissues and it was this antibody which explained the anaphylactic shock. This has been suggested by Malkiel (54) to explain the similar situation which pertains in mice immunized with egg albumin. The spleens, livers and samples of intestine were taken from the mice on day 39 at the conclusion of the experiment. The serosa was removed, the tissues minced and then homogenized in an omnimix. They were subsequently extracted with 0.25 M sucrose. The resulting suspensions were slow frozen and thawed three times to further lyse the cells. These preparations next were centrifuged in a PR-2 centrifuge at 0°C at 5000 RPM for 120 minutes following which the supernatant fluids were removed and inactivated at 56°C for 30 minutes. After a further centrifugation the preparations were stored 0 to 4°C until use; 500 units of penicillin and 500 micrograms of streptomycin were added per ml. CF tests with the tissue extracts with two and five units of complement failed to reveal any fixation of the C'. Ring precipitin tests, Ouchterlony plate

and Preer tube tests were all negative for antibody. The extracts were then cross-tested in Ouchterlony plates with rabbit anti-BSA antiserum. There were strong lines of precipitation produced. This would be expected if the mouse tissue extracts contained residual BSA from the heavy immunization schedule but also suggested the possibility that mouse albumin might be cross-reacting with the rabbit antiserum to BSA. To test this latter contingency spleen, liver, kidney, and intestine was taken from normal non-immunized Swiss mice and tissue extracts prepared as above described. These extracts were then tested in Ouchterlony plates with rabbit anti-BSA. There was strong cross-reactivity and the line of precipitation merged with that produced by the BSA rabbit anti-BSA control in the same plates. This represented good evidence that mouse and bovine albumins are immunologically related. All of the tissues examined gave this phenomenon. The significance of this finding is considered in the Discussion.

DISCUSSION

The importance of genetic constitution as a determinant of antibody production is demonstrated by the results of this simultaneous study of the antibody response of two inbred strains of mice, and the F_1 strains of their reciprocal matings, to two Type A influenza viruses. The immune response as represented by the time curve of antibody production is heritable and its pattern of inheritance indicates this heritability to be of chromosomal origin. It must be mentioned however that ultimate proof of genetic control of antibody production requires the F_2 generation and demonstration of segregation of the observed heritable genetic differences in antibody production. As the entire antibody response was studied, the extent and some of the characteristics of this presumptive genetic control of the immune response can be defined.

It was evident from the results of Part II (Tables 5-8, Figs. 5-8) that the antibody response of the AKR and F_1 strains was virtually identical in titer while the response of the C57BR/cd strain was distinctly different throughout the period of antibody production. This included the period from time of appearance of antibody to its incipient decline. Statistical analysis revealed the similarity of the AKR and F_1 strains to be real and indicated a significant difference

between those three strains and the C57BR/cd animals. In view of this reproduction of the curve of the AKR parent strain by both of the F_1 strains, the entire course of antibody production may be said to be heritable. As reciprocal matings of the parents were employed, it is possible, despite the absence of studies on the F_2 and backcross generations, to rule out non-chromosomal inheritance through the egg cytoplasm of the female parent and environmental influences of the female animal upon the developing embryo and fetus. The parental strains were of a highly homozygous nature, therefore it may be stated in view of this pattern of inheritance that the AKR strain is homozygous for the dominant gene or genes while the C57BR/cd strain is homozygous for the recessive allele or alleles. Possession of the dominant gene or genes determined the maximal titer and also higher titers throughout the time of antibody production. It is also probable that these same genes were involved in the type of immune response engendered, for the AKR and both F_1 strains had not only higher levels of antibody than the C57BR/cd, but their response curves were of a different form than that of the latter strain. The action of these genes is apparently of a quantitative, not qualitative nature as all of the strains were capable of producing antibody of significant titer. This trait is not sex linked and there were no differences in reactivity between the male and female offspring. This latter point is shown by the lack of any subgroups among

the individual titers of the F_1 strains which were of mixed sexes, rather the F_1 titers for any one day appear evenly distributed about the mean for that day for each of the mouse strains.

Thus the maximum antibody response was under probable genetic control, a confirmation of the results of Kleczkowski and Kleczkowska (33) and Sang and Sobey (35). It is apparent that the entire spectrum of the production of antibody was heritable which included the titers on each day, the response to repeated antigenic stimulation as well as distinctive aspects of the curves -- the plateaus and the slopes of development of antibody. However, without the F_2 generation it cannot be determined whether the entire time-course of antibody production was a manifestation of the same gene or genes which determined the maximal response. At the onset of antibody decline the curves separated and statistical significance was lost, presumptive evidence of the cessation of genetic control. The decline of the antibody level was a function of another type of control, as would be expected. There was insufficient data on this aspect of the antibody response in these studies to determine the nature of the antibody decay, it could be under direct genetic control or a manifestation of any number of constitutional factors. Unfortunately there was not enough time available to examine for strain specificity with reference to "decay" of antibody. This could have been determined by measurements of half-life

of passively transferred antibody.

The method of analysis was insufficiently sensitive to detect minor variations, however it is obvious that despite the difference in dosage of the two viruses the slopes, plateaus and general form of the curves for both viruses in each mouse strain were very similar provided actual titers achieved are ignored. Exactly the same observations apply to the CF and HI antibodies to each virus. With reference to this it will be noted that the C57BR/cd strain titers were always one half those of the other strains regardless of the serological test or virus being considered. Thus, not only were the two antibodies to each virus very similar in reactivity if titer was excluded, but the two viruses seemed to generate very analogous responses and this response was true despite the different dosages of the antigens employed. It appears likely, although by no means proved that the same presumed genetic control of the response of the animals to these similar antigens also was in control of the response to antigenic components (the HA and CF antigens) of the complex viral antigen.

This study has demonstrated the heritability of the antibody response, however it is necessary to discuss certain aspects of its possible mechanism. Since the average weight of inbred strains of mice is reproducible from generation to generation, their weight may be said to be a reflection of the genetic constitution of the strains. This being true,

the possibility that the observed antibody response was a function of body weight arises: the smaller strains in receiving a proportionately greater dose of virus might thereby have produced more antibody. This could only have been true here if the weight of the F_1 strains reflected the weight of the AKR strain, whose titers they mimicked. This was not the case. The average weights of the strains were: F_1 of AKR ♀ x C57BR/cd ♂, 20 grams; C57BR/cd, 23 grams; F_1 of C57BR/cd ♀ x AKR ♂, 25 grams; and AKR, 29 grams.

Similarly, differences in metabolic rates among the strains might have accounted for the observed response, causing greater or lesser production and/or destruction of antibody. Wharton et al. (55) have suggested greater destruction of antibody due to an increased metabolic rate to account for low antibody levels in their tumor-bearing mice. Metabolic rate cannot be ruled out as an important factor in these mice because of the lack of data on this subject and the inability to determine its relative importance to antibody production and/or antibody destruction. Only inferences can be made insofar as the metablic rate was reflected in gross differences in activity or excitability of the animals, as judged by casual observation during the 12 months of this study. The AKR strain was the least active strain, while C57BR/cd was significantly more active and the two strains showed greater and lesser titers of antibody

respectively in Part II. This would have been expected were increased metabolic rate accounting for increased destruction of antibody. Further evidence for this came from the half-life studies of Part I where C57BR/cd had an HI antibody half life of 4.4 days and AKR of 6.9 days while the CF half-lives were 2.2 and 4.4 days respectively to the Japan 305/57 virus. However, both F_1 strains were more active than either of the parental strains but produced antibody in an identical fashion to that of the inactive AKR strain. In addition, in Part I, DBA/1, which was the most excitable strain studied, had greater titers of antibody than the other strains to the PR-8 virus in both the HI and CF tests. Likewise, in Part I, the Swiss strain, much more active than AKR, was usually paired in terms of antibody production with that strain. Although metabolic rate cannot be ruled out it is probable that the inherited antibody patterns are a reflection of other aspects of genetic control.

It is not possible to elucidate the origin of the observed genetic differences in the ability to produce antibody. Probably the most obvious theory of these differences is to attribute them to differential rates of maturation of antibody producing stem cells, those strains with rapid maturation rates thereby producing antibody at a faster rate. Or, direct quantitative differences in the ability of mature lymphocytes, or the entire antibody producing tissue, to

produce antibody of a certain level or type could be postulated. Other sites of action such as rates of clearing of antigen from the circulation or length of persistence of antigen in the tissues obviously influence antibody production and may be concerned. Similarly, produced antibody might be held in the tissues, the strains varying in the release of antibody to the circulation. However, the resolution of this interesting and basic problem must await further work.

Throughout this study individual animals and serum pools of a given strain had some variability of titer. Although inbred animals are of a high degree of homozygosity, there will continue to be phenotypic variation between animals (36, 56). This variation is usually less in degree than that of non-selected animals but need not be so (36, 57). Its cause can only be given in generalities and represents the composite effect of many environmental factors, both internal and external acting upon or with the basic genetic predisposition for a certain response. These factors differ slightly between animals in their relative influence and nature. This diversity within strains has been shown in the case of antibody response to correspond to the curve of normal probability (35, 58). The presence of such phenotypic variation does not interfere with the demonstration of heritability of a particular characteristic although it is

present in the progeny as well as the parents of any mating, since, with the aid of statistical analysis one distribution of figures may be compared with another. It might also be said at this point that the rank sum test used in statistical analysis of the results of this experiment does not take into account the fact that the distributions of figures compared conformed to the curve of normal probability. Thus the statistical analysis tended to underestimate the similarities and differences between the strains of mice.

The influenza virus is a highly complex antigenic substance containing representatives of at least five groups of antigens: the neutralizing, flocculating, hemagglutinating, soluble (S) complement-fixing and viral (V) complement-fixing antigens. The Type of the virus is determined by its S antigen and its strain by its hemagglutinating antigen. This study measured the antibody response to the HA antigen and to the viral (V) complement-fixing antigen. Were anti-S rather than anti-V being measured, there would have been cross-reactivity of the C Fantisera which was not observed. In addition, ultracentrifugation removed most of the soluble S antigen and, most relevant, the S antigen usually does not stimulate good production of antibody in an inactivated virus preparation (59, 60).

Although a spaced series of antigen inoculations was given, the antibody response curve in these studies resembled

the response to a single injection of antigen. No anamnestic response was evident. There was one aspect of the response curves which bears further comment. After an initial rise in antibody titer, there was a plateau with time of the maximal titer attained which was maintained until about one week following the cessation of antigenic stimulation; during this period continued doses of antigen served to maintain but not to further increase the titer. This pattern was evident in all of the studies of Parts I and II where the maximum titer was reached prior to the last antigen injection. This type of maximal response to an antigen, an asymptotic type of curve, is a confirmation of a well known immunologic phenomenon (31, 61).

In both Parts I and II the highest titer reached in the HI response to the PR-8 virus was about 1200. Likewise, in both Parts the CF response to either virus achieved a maximal titer of about 80 or 120. This suggests that there is a maximal attainable titer for both the HI and CF antibodies to each of these viruses which is independent of dosage as the viral dose in Part I and Part II was different.

It was not possible to account for the changed reactivity in the HI test of the C57BR/cd and AKR strains of mice in Parts I and II. In Part I the C57BR/cd animals tended to have higher HI titers than the AKR strain during

the first part of the antibody curves, while in Part II this relationship was reversed. Age has been shown by Fink (26) and Overman (62) to influence antibody response. The age of the strains at the onset of the experiment in Part I were: C57BR/cd, three months and AKR, four months; and in Part II were: C57BR/cd five months and AKR, five months. It is difficult to see how such small age differences among adult mice should influence antibody production, however Fink (26) found very large differences in antibody production in mice of two and four months of age, both ages being presumably adult from the standpoint of immunological maturity. There was one year between Part I and Part II however the caging and treatment of the mice was identical in both portions of the study. It is very unlikely that a differentiation of one of the mouse strains to a subline occurred during this period of time. The virus antigens used in Part II were the direct descendents of those of Part I, but a slight change might have occurred with egg passage. The differences in reactivity were evident only in the HI test, which is less reproducible and more susceptible to non-specific influences than is the CF test. Although unexplained, this change in reactivity did not obviate the results since within Parts I and II the findings were entirely consistent.

The measure of dosage used in an attempt to equate the dose of virus in Parts I and II, the "equivalent EID_{50}", was

evidently fallacious as the generated antibody response in the two halves of the experiment did not bear any relationship to this measure of dosage. Although the EID_{50} does bear a relationship to the mouse infectivity dose in the case of live influenza viruses (63), this is not true with inactivated virus. This is a further confirmation of the fact that natural resistance and antibody production are based upon different factors. It likewise may be observed from study of the HA doses of Parts I and II for each virus that the antibody response is not in direct relationship with this measure of dosage either.

The production of anaphylactic shock with BSA in this investigation is in agreement with the work of Solotorovsky (46), Cameron (47) and Terres (64) who likewise noted this phenomenon. The occurrence of anaphylactic shock represented evidence for the possession of anti-BSA by these mice. Solotorovsky (46) observed only small amounts of circulating antibody in his animals while Benedict (48) and Terres (64) found rather large amounts of circulating antibody in BSA immunized mice. In the present study no circulating antibody nor any tissue antibody was demonstrable by any of multiple serological procedures. Although it is unlikely that these mice possessed circulating antibody the inability to demonstrate tissue antibody does not mitigate against its presence.

A very interesting result of this work was the observation

that rabbit anti-BSA cross-reacted in Ouchterlony plates with normal, non-immunized mouse tissue extracts, and that the line of precipitation produced was continuous with that of the BSA rabbit anti-BSA control. As emphasized by Maurer (65) and Cohn (66), it is necessary to be extremely careful in interpreting the results of such cross-reactions; Wilson (67) has mentioned the hazards of studying such reactions in Ouchterlony plates. These cross-reactions, although not spurious, are influenced in degree by many factors, the most important of which is the length of immunization which is in direct proportion with the species cross-reactivity of the antibody produced (68, 69). Despite these cautions the demonstration of the observed mouse-bovine albumin immunological relationship furnishes a convenient explanation of the lack of circulating antibody and the observed anaphylactic shock in the mice studied. One may postulate the theory that the injected BSA, being similar to the circulating and tissue mouse albumin, stimulated antibody production to only a portion of the protein, the dissimilar component. Hooker has also suggested multiple antibody production to a single homogenous antigen (70). The produced antibody would then have been adsorbed onto the albumin of the serum and of the mouse tissues being rendered thereby non-detectable. After assimilation during the rest period, reinjected BSA then combined with this anti-albumin in the tissues and it was this reaction which was responsible for

the anaphylactic shock.

This cross-reacting system may be added to several other known inter-species immunologically related albumins. Mouse and human albumins have been shown by Clausen (71) to cross-react immunologically as have human and bovine albumins by Melcher (72), Maurer (65) and Naylor (73). Human and horse albumin have also been shown to cross-react by Adair (68).

SUMMARY

The entire time-course of antibody production was studied in four inbred strains of mice and the F_1 progeny of reciprocal matings of two of the strains. Two inactivated Type A influenza viruses were given as antigens in a series of multiple inoculations and the antibody response was analyzed by both the hemagglutination-inhibition and complement-fixation techniques. The time of antibody appearance, its slope of production, the time and amount of maximum response, the slope of decline and general characteristics of the antibody curves were studied and the pattern of inheritance of these elements of the immune response in the F_1 strains was delineated.

The results of this study demonstrated genetic heritability of the immune response and represented presumptive evidence for genetic control of antibody production although the absolute demonstration of control lies in the study of the segregation of the observed heritable patterns in the F_2 generation. The entire curve of production of antibody to the time of incipient decline of the antibody level was found to be chromosomally inherited. One strain of mice, AKR, was homozygous for the dominant gene or genes while another strain, C57BR/cd carried the homozygous recessive allele. Dominance was manifest in higher titers and a distinctive curve of antibody response. The genes

controlling the maximum response and the total immune response appeared as well to function in the determination of the hemagglutination-inhibition and complement-fixation response to both of the closely related viruses. The implications of these findings are discussed.

In addition, the response of the mice to bovine serum albumin (BSA) was investigated. Although originally intended as a third antigen for characterization of the immune response, several protocols of intensive antigenic stimulation failed to elicit antibody detectable either in the serum or tissues by multiple serological techniques. However, following a lapse of injections of antigen, reinoculation of BSA resulted in anaphylactic shock in the mice. Investigations into the cause of the shock and of the absence of antibody ultimately revealed the presence of an immunological cross-reacting system in bovine and mouse albumins. It is presumed that the mice produced antibody to only a portion of the injected antigenically similar albumin, that this antibody was quickly adsorbed onto mouse albumin in the tissues and that the reinjection of BSA resulted in antigen-antibody reactions in the tissues producing anaphylactic shock.

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TABLE 1. PART I: HEMAGGLUTINATION-INHIBITION TITERS TO PR-8 VIRUS

	DAY 4		7		14		21		28		35		42	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
AKR	320 320 320	320	240 240 240	240	240 240 240	240	600 800 800	727	600 600 600	600	600 600 600	600	300 300 300	300
C57BR/cd	120 120 120	120	320 320 320	320	960 960 960	960	1200 800 600	832	600 800 800	727	600 600 600	600	300 300 300	300
DBA/1	120 120 120	120	480 480 480	480	960 960 960	960	1200 1200 1200	1200	1200 1200 800	1048	1200 1200 1200	1200	600 600 400	524
SWISS	240 240 240	240	160 160 160	160	240 480 240	302	600 600 600	600	600 800 800	727	600 600 600	600	600 600 600	600
CONTROL	4000	4000	4000	4000	6000	6000	6000	6000	4000	4000	4000	4000	3000	3000

On the left are given the strains of mice. The control was a chicken antiserum retitrated on each day of analysis. Day refers to the day of bleeding. Column A gives the titers of the three serum pools of each mouse strain. Column B gives the geometrical mean titer of the titers of the serum pools given in column A.

TABLE 2. PART I: HEMAGGLUTINATION-INHIBITION TITERS TO JAPAN 305/57 VIRUS

	DAY 4		7		14		21		28		35		42	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
AKR	<10		< 10		40		90		160		70		40	
	<10	<10	< 10	<10	30	36	90	99	160	145	100	79	30	36
	<10		< 10		40		120		120		70		40	
C57BR/cd	<10		10	10	80		120		240		100		30	
	<10	<10	10		80	80	120	120	240	240	80	86	30	30
	<10		10		80		120		240		80		30	
DBA/1	<10		10		60		120		160		180		80	
	<10	<10	10	10	80	73	90	99	160	160	120	137	80	73
	<10		10		80		90		160		120		60	
SWISS	<10		< 10	<10	30		120		80		40		30	
	<10	<10	< 10		40	33	120	109	80	73	40	46	30	30
	<10		< 10		30		90		60		60		30	
CONTROL	300	300	200	200	300	300	200	200	300	300	300	300	300	300

Legend as in Table 1

TABLE 3. PART I: COMPLEMENT-FIXATION TITERS TO PR-8 VIRUS

	DAY 4		7		14		21		28		35		42	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
AKR	< 10		10		40		100		270		75		35	
	< 10	< 10	20	16	80	64	100	106	270	236	100	83	35	33
	< 10		20		80		120		180		75		30	
C57BR/cd	< 10		10		120		80		135		63		20	
	< 10	< 10	10	10	120	120	80	80	135	135	63	63	20	20
	< 10		10		120		80		135		63		20	
DBA/1	< 10		40		160		160		180		150		40	
	< 10	< 10	30	36	160	160	140	153	270	206	150	131	40	40
	< 10		40		160		160		180		100		40	
SWISS	< 10		10		40		120		135		125		80	
	< 10	< 10	10	10	40	50	80	92	180	164	88	111	60	73
	< 10		10		80		80		180		125		80	
CONTROL	135	135	135	135	90	90	135	135	135	135	90	90	90	90

Legend as in Table 1 except the control here was a mouse antiserum to the virus.

TABLE 4. PART I: COMPLEMENT-FIXATION TITERS TO JAPAN 305/57 VIRUS

	DAY 4		7		14		21		28		35		42	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
AKR	< 10		< 10	< 10	60		60		120		38		15	
	< 10	< 10	< 10		60	60	60	66	120	120	45	40	15	13
	< 10		< 10		60		80		120		38		10	
C57BR/cd	< 10		< 10		30		30		60		10		< 10	
	< 10	< 10	< 10	< 10	15	24	30	30	40	52	10	10	< 10	< 10
	< 10		< 10		30		30		60		10		< 10	
DBA/1	< 10		< 10		20		30		40		10		< 10	
	< 10	< 10	< 10	< 10	20	20	25	28	60	52	10	10	< 10	< 10
	< 10		< 10		20		30		60		10		< 10	
SWISS	< 10		< 10		50		100		120		45		15	
	< 10	< 10	< 10	< 10	50	50	120	106	120	120	38	42	10	13
	< 10		< 10		50		100		120		45		15	
CONTROL	100	100	120	120	120	120	120	120	120	120	90	90	120	120

Legend as in Table 1 except the control here was a mouse antiserum to the virus.

TABLE 5. PART II: HEMAGGLUTINATION-INHIBITION TITERS TO PR-8 VIRUS

	DAY 4		DAY 7		DAY 14		DAY 21		DAY 28	
	A	B	A	B	A	B	A	B	A	B
AKR	40 60 40 30 30 40	39	300 300 300 300 300	300	960 1280 960 1920 1280	1244	1200 1200 1200 800 1600	1172	1280 960 960 640	932
C57BR/cd	10 10 < 10 10 20 10	8	800 600 800 800 600 800	727	640 640 640 640 640	640	600 400 400 300 300	387	240 240 240 240 240	240
F_1 AKR ♀ × C57BR/cd ♂	40 40 40 40 40 40	40	300 300 300 300 300 200	280	1280 960 960 1280 1920	1237	1200 1200 1200	1200	480 320 640	462
F_1 C57BR/cd ♀ × AKR ♂	40 40 60 30 40 30	39	300 200 300 300 300 200	262	1920 1920 1280 1280 1280	1505	1200 1200 1200 1200	1200	960 960 960 960	960
CONTROL	3000	3000	4000	4000	4000	4000	3000	3000	3000	3000

On the left are given the strains of mice. The control was a chicken antiserum retitrated on each day of analysis. Day refers to the day of bleeding. Column A gives the titers of the individual mice of each mouse strain. Column B gives the geometrical mean titer of the titers of the individual mice given in Column A.

TABLE 6. PART II: HEMAGGLUTINATION-INHIBITION TITERS TO JAPAN 305/57 VIRUS

	DAY 4		7		14		21		28	
	A	B	A	B	A	B	A	B	A	B
AKR	< 10		40		640		400		640	
	< 10		30		640		600		640	
	< 10	< 10	30	33	960	622	800	583	640	640
	< 10		30		480		600		640	
	< 10		30		640					
	< 10		40		480					
C57BR/cd	< 10		80		160		200		160	
	< 10		60		160		400		160	
	< 10	< 10	80	69	240	231	300	293	160	160
	< 10		60		320		300		160	
	< 10		60		240		300		160	
	< 10		80		320					
F_1 AKR ♀ + C57BR/cd ♂	< 10		40		640		600		240	
	< 10		30		480		600		240	
	< 10	< 10	40	36	640	604	600	600	240	240
	< 10		30		640		600		240	
	< 10		40		640		600		240	
	< 10		40							
F_1 C57BR/cd ♀ + AKR ♂	< 10		30		480		600		320	
	< 10		20		480		600		640	
	< 10	< 10	40	29	640	528	800	635	480	462
	< 10		30		480		600			
	< 10		30		480		600			
	< 10		30		640					
CONTROL	300	300	300	300	200	200	300	300	300	300

Legend as in Table 5

TABLE 7. PART II: COMPLEMENT-FIXATION TITERS TO PR-8 VIRUS

	DAY 4		7		14		21		28	
	A	B	A	B	A	B	A	B	A	B
AKR	< 5	< 5	5	6	60	60	75	73	80	80
	< 5		5		60		50		80	
	< 5		5		60		75		80	
	< 5		7½		60		100		80	
	< 5		5		60		75			
	< 5		7½							
C57BR/cd	< 5	< 5	< 5	2	20	29	25	36	10	10
	< 5		5		30		25		15	
	< 5		< 5		30		38		10	
	< 5		< 5		40		50		8	
	< 5		5		30		50		8	
	< 5		< 5							
F1 AKR ♀ × C57BR/cd ♂	< 5	< 5	7½	7	80	55	50	57	40	40
	< 5		7½		60		50		40	
	< 5		7½		60		75		40	
	< 5		7½		60					
	< 5		5		30					
	< 5		7½							
F1 C57BR/cd ♀ × AKR ♂	< 5	< 5	5	6	60	71	75	75	60	58
	< 5		7½		80		75		40	
	< 5		5		80		75		80	
	< 5		5		80		75		60	
	< 5		7½		60					
	< 5		7½							
CONTROL	200	200	150	150	200	200	300	300	300	300

Legend as in Table 5 except the control here was a mouse antiserum to the virus.

TABLE 8. PART II: COMPLEMENT-FIXATION TITERS TO JAPAN 305/57 VIRUS

	DAY 4		7		14		21		28	
	A	B	A	B	A	B	A	B	A	B
AKR	<5		<5		80		75		80	
	<5		5		60		75		80	
	<5		5		120		75		80	
	<5	<5	<5	3	80	78	75	75	80	80
	<5		5		60					
	<5		5		80					
C57BR/cd	<5		<5		30		38		15	
	<5		<5		20		38		30	
	<5	<5	<5	<5	20	19	25	37	20	19
	<5		<5		15		50		20	
	<5		<5		15		38		15	
	<5		<5		20					
F_1 AKR ♀ + C57BR/cd ♂	<5		10		80		50		60	
	<5		10		60		75		60	
	<5		7½		60		75		60	
	<5	<5	10	9	80	71	75	69	60	60
	<5		7½		80		75		60	
	<5		10							
F_1 C57BR/cd ♀ + AKR ♂	<5		5		80		50		40	
	<5		<5		40		75		40	
	<5		5		60		50		40	
	<5	<5	<5	2	80	65	100	62		40
	<5		<5		60		50			
	<5		<5		80					
CONTROL	200	200	150	150	100	100	150	150	200	200

Legend as in Table 5 except the control here was a mouse antiserum to the virus.

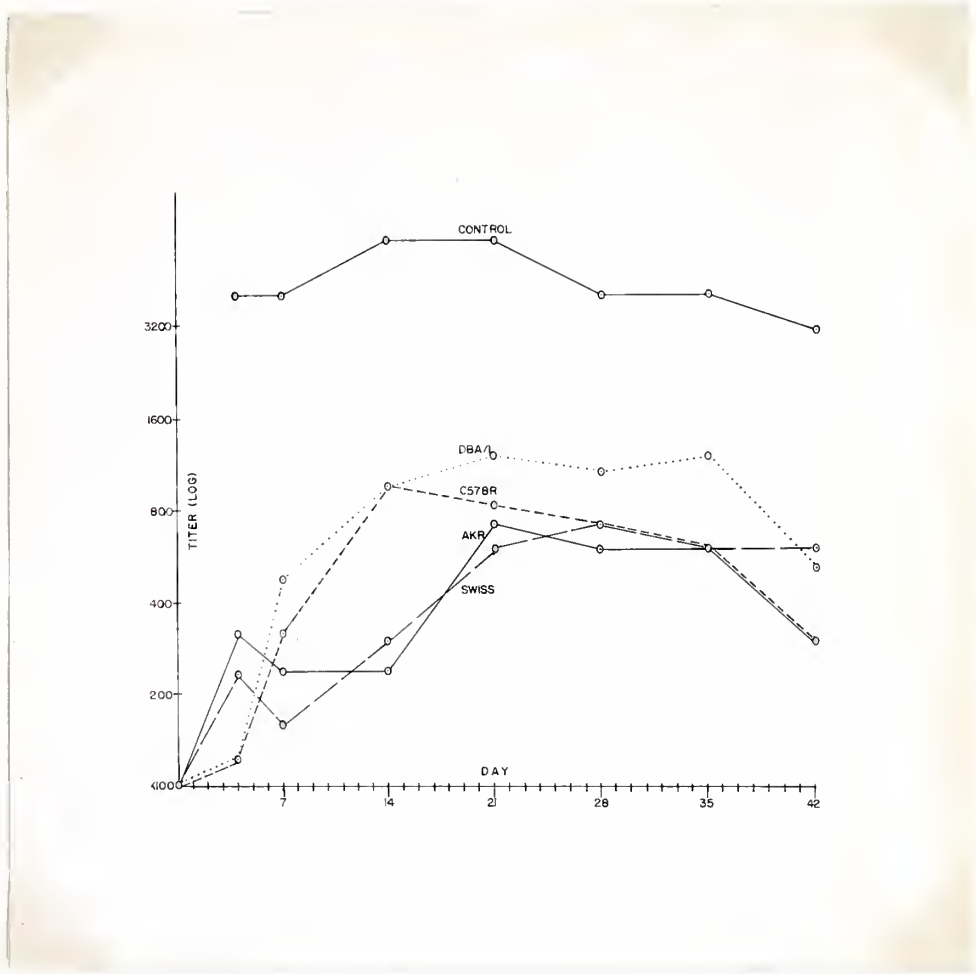

FIGURE 1

Part I: Hemagglutination-inhibition antibody response curve to PR-8 virus. The control represents a series of repeated titrations of the same serum, it is not a curve of antibody production.

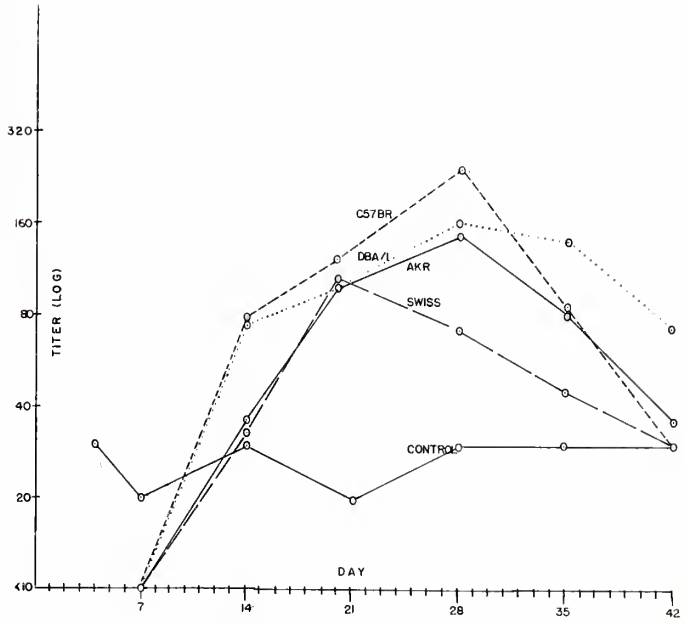

FIGURE 2

Part I: Hemagglutionation-inhibition antibody response curve to Japan 305/57 virus. The control represents a series of repeated titrations of the same serum, it is not a curve of antibody production. For convenience, the control is plotted one log below actual titer.

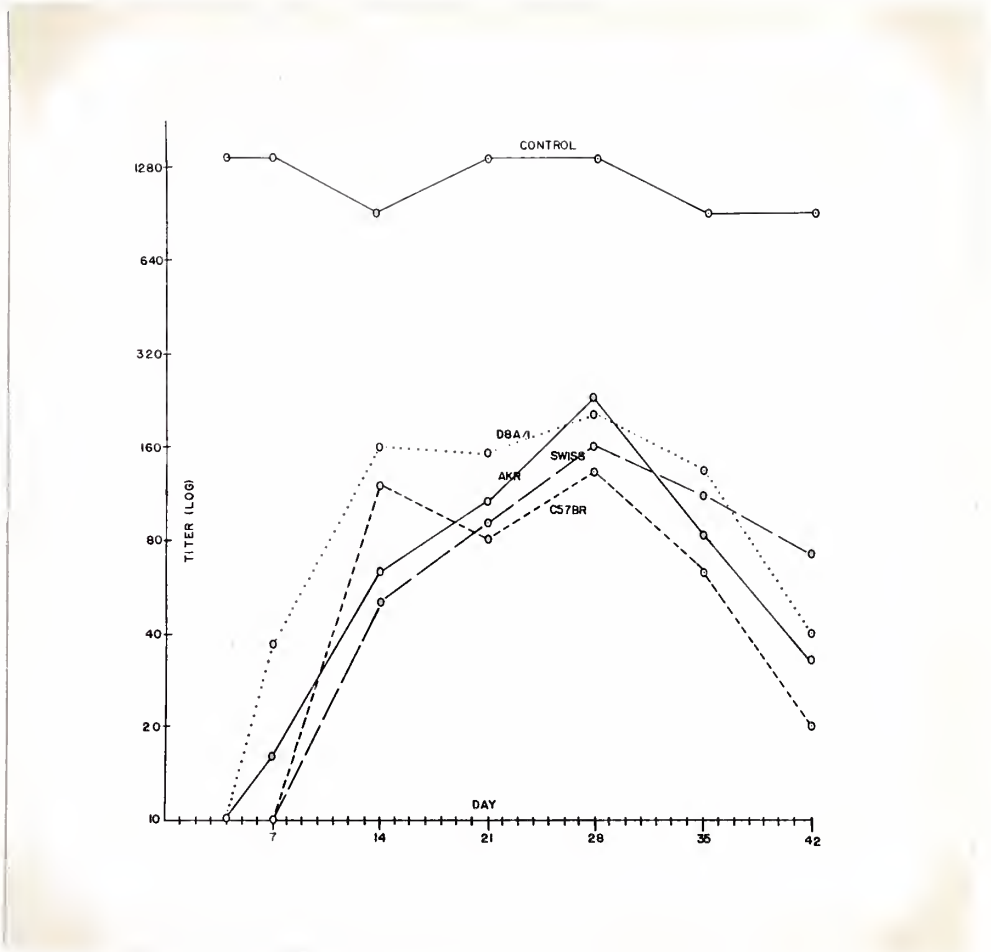

FIGURE 3

Part I: Complement-fixation antibody response curve to PR-8 virus. The control represents a series of repeated titrations of the same serum, it is not a curve of antibody production. For convenience, the control is plotted one log above actual titer.

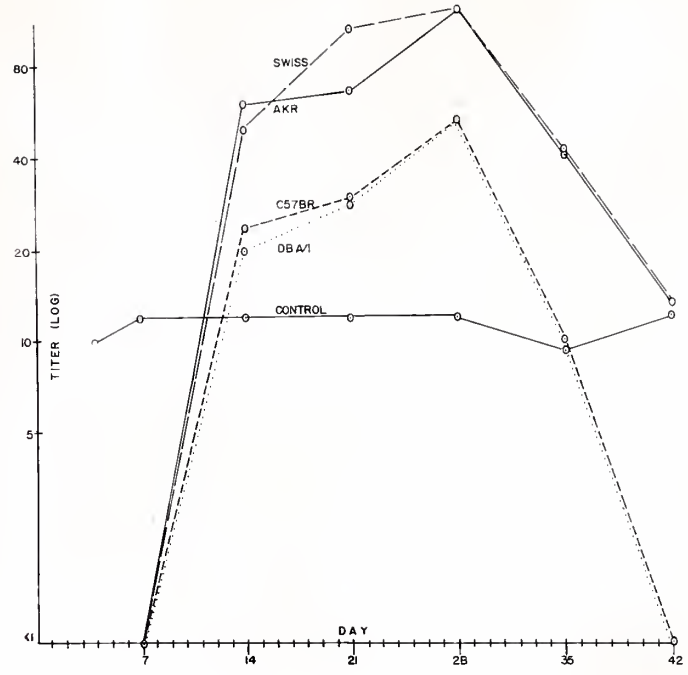

FIGURE 4

Part I: Complement-fixation antibody response curve to Japan 305/57 virus. The control represents a series of repeated titrations of the same serum, it is not a curve of antibody production. The control is plotted one log below actual titer.

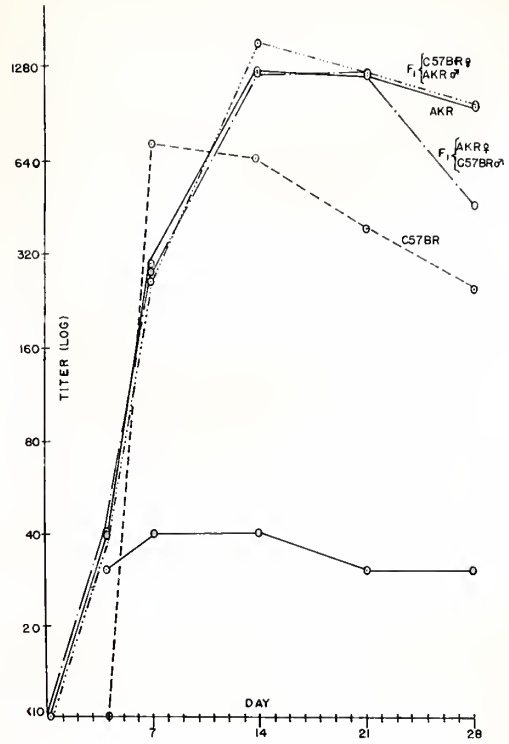

FIGURE 5

Part II: Hemagglutination-inhibition antibody response curve to PR-8 virus. The control represents a series of repeated titrations of the same serum, it is not a curve of antibody production. For convenience, the control is plotted two logs below actual titer.

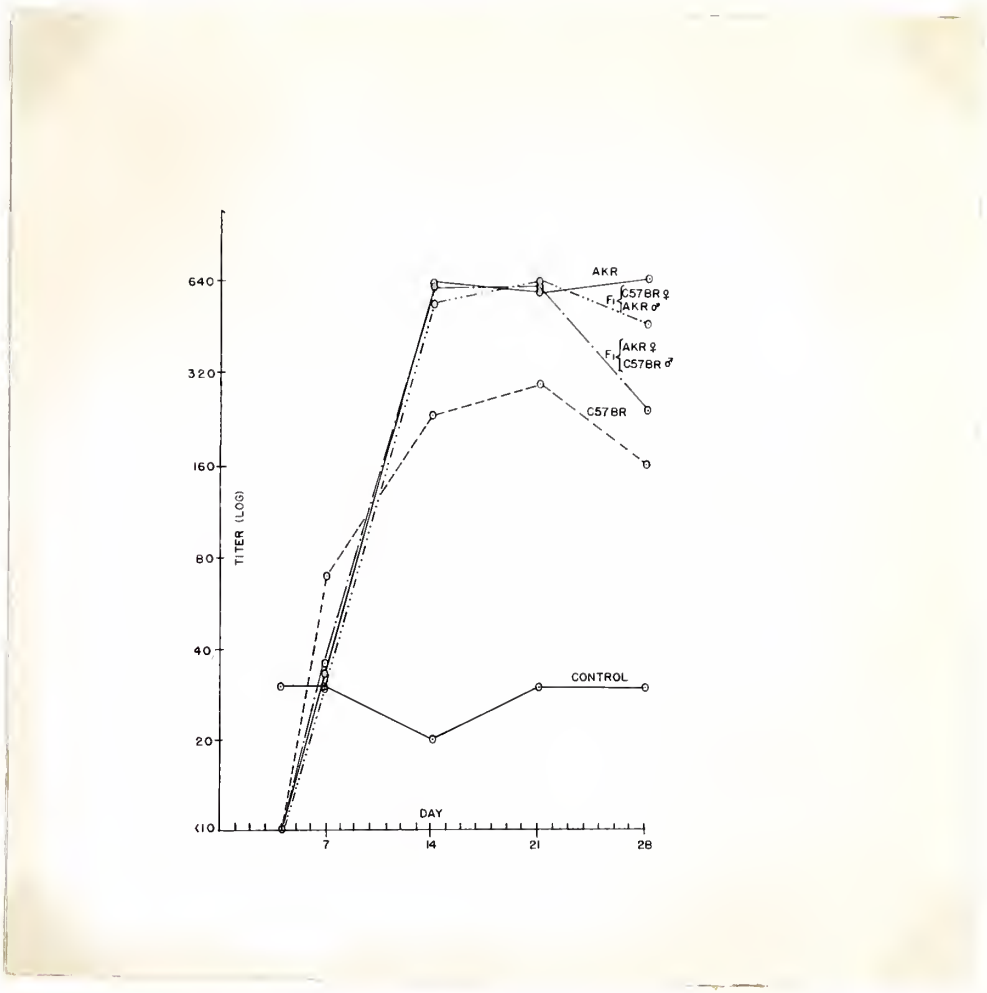

FIGURE 6

Part II: Hemagglutination-inhibition antibody response curve to Japan 305/57 virus. The control represents a series of repeated titrations of the same serum, it is not a curve of antibody production. For convenience, the control is plotted one log below actual titer.

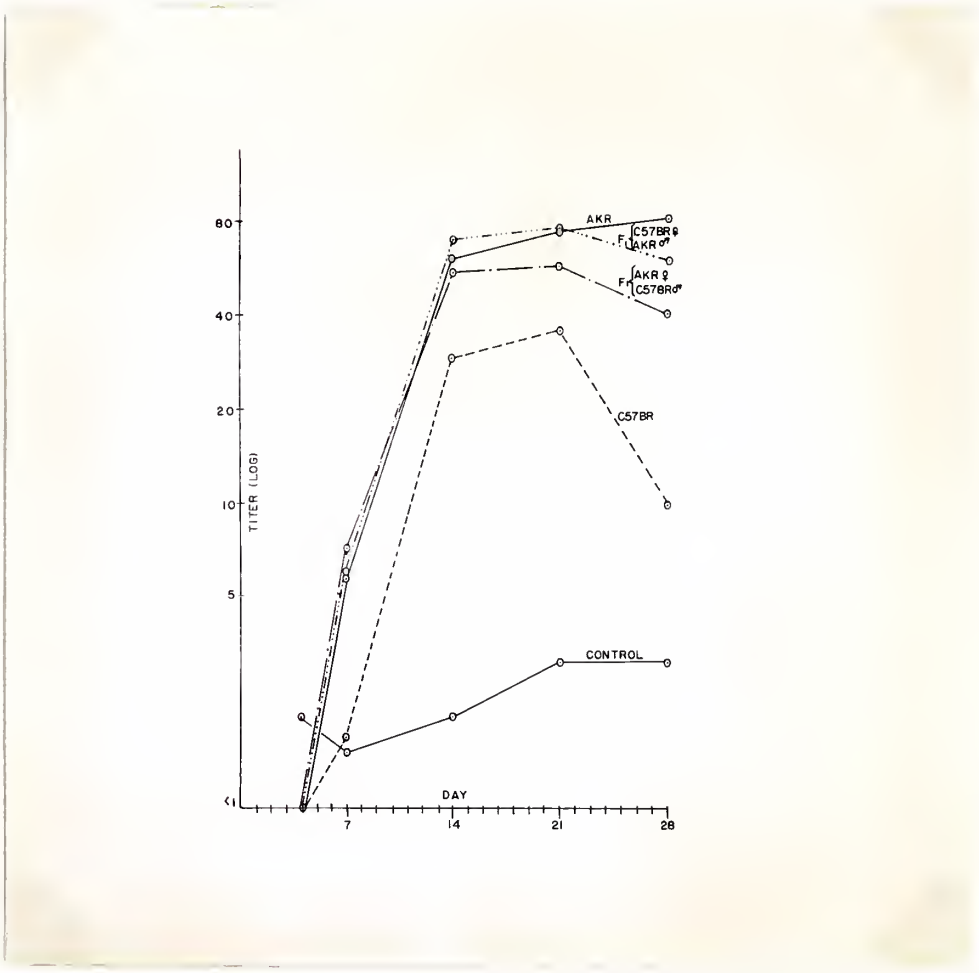

FIGURE 7

Part II: Complement-fixation antibody response curve to PR-8 virus. The control represents a series of repeated titrations of the same serum, it is not a curve of antibody production. For convenience, the control is plotted two logs below actual titer.

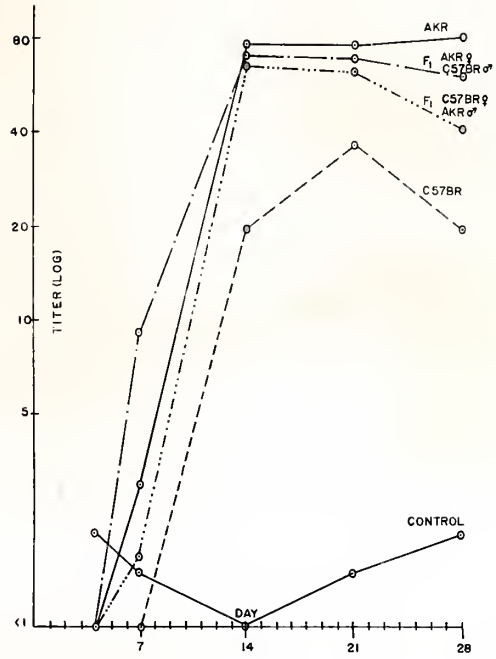

FIGURE 8

Part II: Complement-fixation antibody response curve to Japan 305/57 virus. The control represents a series of repeated titrations of the same serum, it is not a curve of antibody production. For convenience, the control is plotted two logs below actual titer.

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