GENETIC CONTROL OF THE ANTIBODY RESPONSE

I. Demonstration of Determinant-Specific Differences in Response to Synthetic Polypeptide Antigens in Two Strains of Inbred Mice

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There is considerable evidence from a variety of sources to indicate that the immune response is under some form of genetic control (1–13). Most previous attempts to demonstrate genetic control of the antibody response have succeeded only in showing a definite correlation between the quantitative response of parents and offspring, without any clearcut pattern of inheritance (2, 3, 5, 7). The only exception to this statement is the finding by Levine, Ojeda, and Benacerraf (9, 10, 12) that the response of guinea pigs to any of four different hapten-poly-L-lysine conjugates appears to be genetically transmitted as a unigenic Mendelian dominant trait, the homozygous recessive animals being unable to respond to any of the four conjugates tested. While the precise nature of this qualitative genetic control remains unknown (11), the specificity of the genetic difference for the poly-L-lysine "carrier" and not for the hapten determinant, has led the authors to conclude that this control is exercised at some point prior to the actual induction of antibody formation. Up to the present time, it has not been possible to demonstrate genetic control of the production of antibody directed against a particular antigenic determinant or group of determinants.

The present studies were begun following the chance observation, in animals immunized by Dr. John Humphrey, of a marked quantitative difference between two strains of rabbits (Sandylops and Himalayans) in their antibody response to a branched, multichain synthetic polypeptide, poly(tyr,glu)-poly-DL-ala--polylys, (T,G)-A--L. This finding stimulated a search for a similar difference in antibody response between strains of inbred mice, and led to the discovery that CBA and C57 mice also differ markedly in their antibody response to (T,G)-A--L. The results indicate that this difference is about tenfold in magnitude, genetically determined, and specific for the antigenic determinant. The genetic disparity underlying this difference in antibody response

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appears to consist of a single major genetic factor, with perhaps one or more modifying factors whose quantitative contribution is minor.

Materials and Methods

Antigens.—Most of the studies were carried out using (T,G)-A--L 509 as the antigen. This polypeptide is synthesized in three steps, and consists of a poly-L-lysine backbone, with side chains of poly-DL-alanine built on the ε-amino groups of lysine. The amino termini of these poly-DL-alanine side chains are then covered with peptides containing both L-tyrosine and L-glutamic acid. The general plan of this antigen is shown in Fig. 1. The residue molar ratio in the polymer 509 was lys:tyr:glu:DL-ala as 1:2.14:4.1:19.6. Its average molecular weight, 232,000, was calculated from an intrinsic sedimentation coefficient, s_{20w} = 7.5S, an intrinsic diffusion coefficient, $D_{20w} = 2.5 \times 10^{-7}$ cm^2/second, and a partial specific volume,

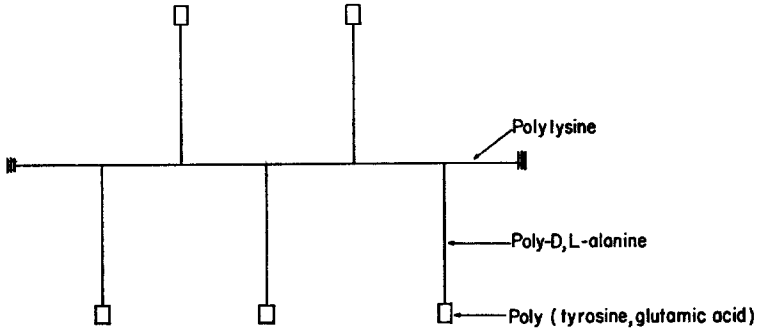

FIG. 1. A schematic diagram of the structural pattern of (T,G)-A--L 509.

$\bar{v}$ = 0.685. The sedimentation and diffusion coefficients were measured in 0.1 M phosphate buffer pH 7.0, in a Spinco model E ultracentrifuge, as described by Sela *et al.* (18). These figures correspond to an average of 100 lysine residues in the polylysine backbone; 17 alanine residues in each side chain; and 1 tyrosine and 2 glutamic residues on the tip of each side chain. This sample was prepared and characterized by Dr. Israel Schechter.

(H,G)-A--L 220 is a synthetic polypeptide built on the same general pattern as (T,G)-A--L, but with L-histidine substituted for tyrosine. The residue molar ratio in polymer 220 was lys:his:glu:DL-ala as 1:1.10:3.7:18.8, and its average molecular weight is 40,000.

Animals.—CBA and C57 black mice were those available at the National Institute for Medical Research, Mill Hill. These strains had been maintained at the Institute for a number of years. All other strain combinations were bred from these two strains.

Immunization.—Considerable effort was spent in determining the route and mode of immunization which was most effective. (T,G)-A--L 509 proved to be non-antigenic if given in 0.15 N NaCl, pH 7.0 (saline) or adsorbed on alum, weakly antigenic when given in incomplete Freund's adjuvant, and moderately antigenic in complete Freund's adjuvant. Foot-pad injections gave titers slightly higher than the intraperitoneal or intramuscular route. Accordingly, mice were immunized with 1, 10, or 100 μg of antigen in complete Freund's adjuvant (1 part antigen: 1 part lanolin: 2 parts liquid paraffin, with 4 mg *Mycobacterium tuberculosis* per ml) distributed in the two hind foot-pads, followed 5 weeks later by an identical injection of the antigen in saline. All mice were bled from the tail vein on the 10th day after the booster injection.

Serum Antibody Determinations.—All sera were titered individually. Ten μl of mouse antiserum was incubated with 1.6 μg of (T,G)-A--L 509 trace labeled with I^{125} by the method of McFarlane (14), or with 0.14 to 0.20 μg of (H,G)-A--L 220 trace labeled with I^{131} by the method of Greenwood, Hunter, and Glover (15), or with 20 μg of bovine serum albumin (BSA) (Armour Pharmaceutical Company, Kankakee, Illinois), labeled with I^{131} by the method of McFarlane (14), in a volume of 0.5 to 0.75 ml for 1 hour at 37°C. Then, an excess of rabbit anti-mouse γ-globulin antiserum was added in an amount sufficient to precipitate all the mouse antibody present. After overnight incubation at 4°C, the precipitates were centri-

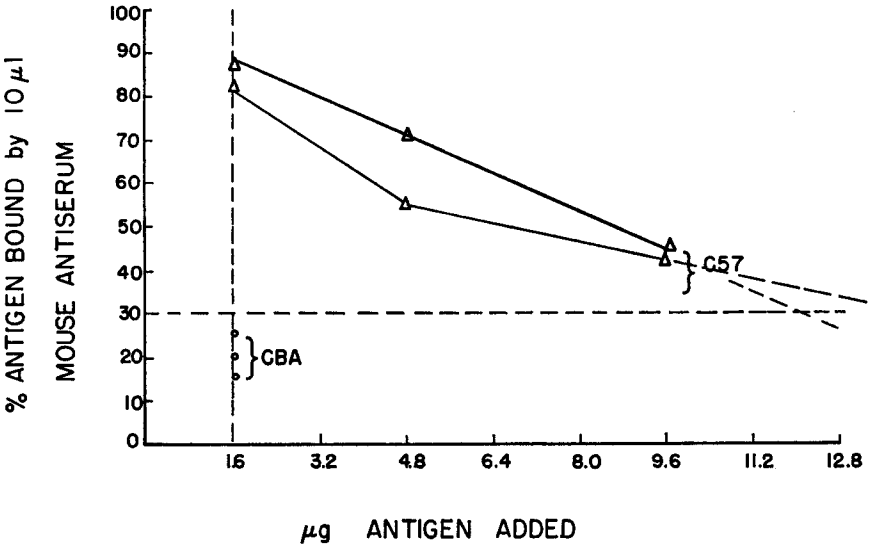

FIG. 2. Alternative methods of comparing various mouse antisera with respect to antigen-binding capacity; (*a*) comparison of "per cent antigen bound" at a fixed amount of antigen/volume mouse antiserum (vertical dotted line), and (*b*) comparison of "amount antigen bound" (per cent × total antigen) at a fixed per cent antigen bound of 30 per cent (horizontal dotted line). On the latter basis, 3 CBA's bind about 32 μg antigen/ml antiserum, and two C57's bind about 340 to 380 μg antigen/ml antiserum.

fuged, washed once, and the radioactivity in precipitate and supernatant counted in a well-type scintillation counter. Results were expressed as "per cent antigen bound", *i.e.* the percentage of 1.6 μg of (T,G)-A--L 509 bound by 10 μl of mouse antiserum. Ideally, sera should be compared in terms of antigen-binding capacity at a fixed value for per cent antigen bound, such as 30 per cent. However, this requires a series of determinations on each serum, and a large volume of rabbit anti-mouse γ-globulin. The alternative method of comparing per cent antigen bound at a fixed amount of antigen/volume mouse antiserum is more economical, and only has the effect of minimizing differences between various sera, as can be seen in Fig. 2. Normal mouse serum (either CBA, C57, or F_1) usually binds only 1 to 2 per cent in this system, and never more than 5 per cent. The iodinated antigen is 96 per cent precipitable by specific antibody.

The rabbit anti-mouse γ-globulin used in these experiments was obtained by immunizing rabbits with a partially purified 7S γ_2 mouse myeloma protein (MP 5563). This antiserum

reacted with purified mouse Bence-Jones protein, 7S γ_1-globulin, 7S γ_2-globulin, and β_2a (IgA) globulin indicating that the antiserum reacted with determinants common to most, if not all, mouse immunoglobulins. In addition, identical values for per cent antigen bound were obtained using this antiserum or a polyvalent antiserum reacting with the four major classes of immunoglobulins: 7S γ_1, 7S γ_2, β_2a (IgA), and γ_1M (IgM) (16). These studies characterizing the rabbit anti-mouse γ-globulin were performed with the very kind collaboration of Dr. John L. Fahey.

RESULTS

Preliminary experiments employing CBA, C57, CBA × C57 F_1, F_1 × CBA, and F_1 × C57 mice (65 animals) gave strong indications of a genetic factor responsible for the large and consistent difference in antibody response between the two parent strains of mice. These experiments were then repeated at varying doses of antigen, and in larger numbers of animals.

(*a*) Immunization with 10 μg (T,G)-A--L 509 in Freund's adjuvant and 10 μg in saline.

These results are shown in Fig. 3, where the number of animals falling in a given percentile of "per cent antigen bound" is plotted for the various strains and strain combinations. CBA's give a very poor response to this antigen, while C57's are much stronger responders. Individual titrations of C57 antisera have shown that their antigen-binding capacity at comparable levels of per cent antigen bound is tenfold or more greater than the antigen-binding capacity of CBA antisera. The most striking points are the uniformity of response within the CBA or C57 strains; the large difference between the strains; and the lack of any overlap. The CBA × C57 F_1 mice give a response intermediate between the parental strains and slightly overlapping the C57 strain. F_1 × CBA back-cross mice all fall in the range encompassed by the F_1 or the CBA mice while F_1 × C57 back-cross mice all fall within the range encompassed by the F_1 or C57 mice. Again, there is a striking absence of overlap. Antibody response was not linked to sex or coat color. To rule out the possibility that these differences reflected different points on a dose-response curve, further immunization studies were done.

(*b*) Immunization with 1 μg (T,G)-A--L 509 in Freund's adjuvant and 1 μg in saline.

These results are plotted in Fig. 4. It is immediately apparent that this dose of antigen is submaximal in both CBA and C57 mice. In both strains the animals either show no detectable response or a very low response, and both strains are below their average response to 10 μg of antigen.

(*c*) Immunization with 100 μg of (T,G)-A--L 509 in Freund's adjuvant and 100 μg in saline.

These results are plotted in Fig. 5. In general, there is slightly greater variability than with the 10 μg dose, but the overall pattern remains the same. The exception to this is the lower response of the C57 mice. The reason for

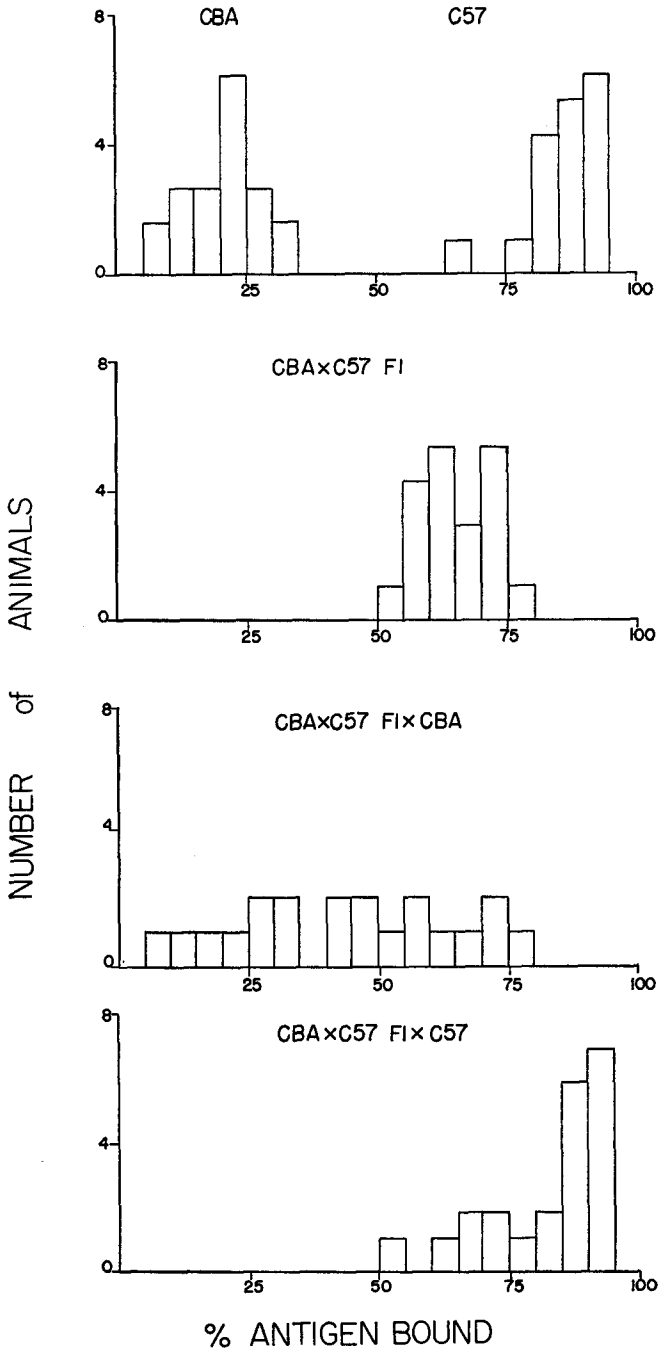

FIG. 3. Immune response of mice given 10 μg (T,G)-A--L 509 in complete Freund's adjuvant, and boosted with 10 μg of the same antigen in saline.

this is not clear. However, prior to boosting, the 100 μg C57 mice have a higher titer than the 10 μg C57 mice, and their lower response to boosting apparently indicates that 100 μg of antigen in saline is an overdose, for reasons which are

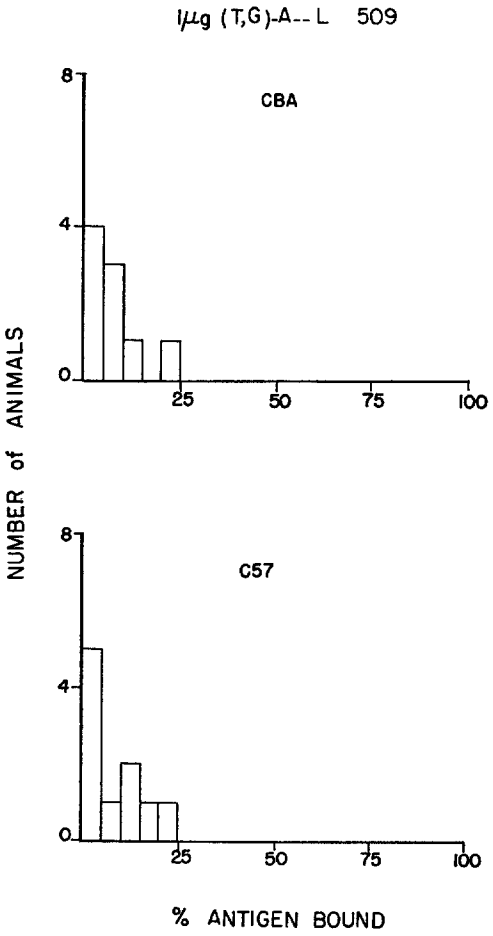

FIG. 4. Immune response of mice given 1 μg (T,G)-A--L 509 in complete Freund's adjuvant, and boosted with 1 μg of the same antigen in saline.

as yet unknown. The fact remains that a tenfold increase in dose of antigen does not abolish the strain differences in response which are seen with a 10 μg dose.

The possibility remains that this disparity in antibody response reflects some non-specific factor such as nutrition, environmental effect, or age. All the mice used were of the same age (2 to 3 months at the start of immuniza-

tion), both sexes (in equal numbers) and were handled in an identical manner. In addition, breeding of the F_1 and back-cross combinations necessitated mixing the strains, so that their bacterial flora were presumably similar.

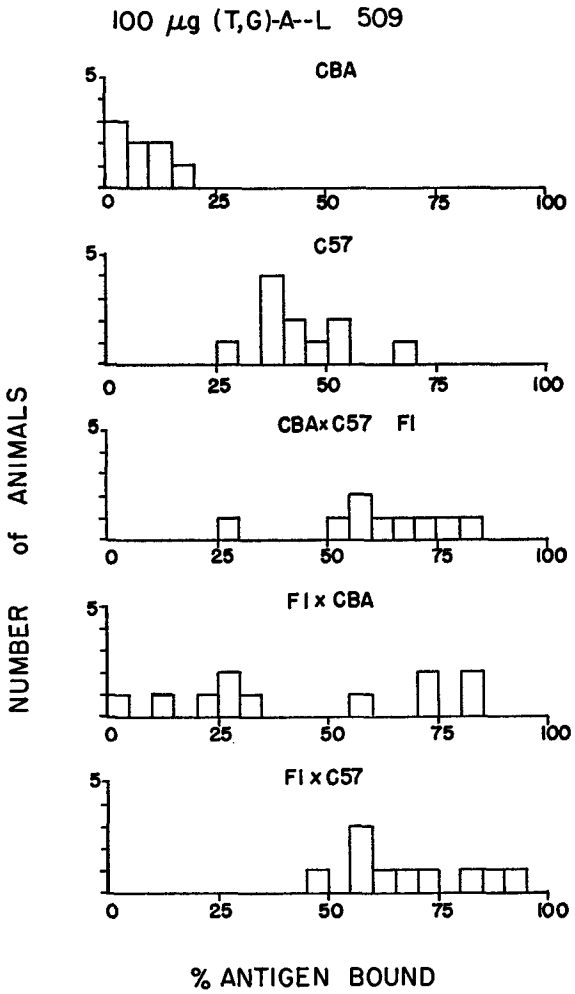

FIG. 5. Immune response of mice given 100 μg of (T,G)-A--L 509 in complete Freund's adjuvant, and boosted with 100 μg of the same antigen in saline.

It is also possible that the observed differences in immune response reflect some general difference in immunologic reactivity, such as a difference in total number of lymphoid cells, or in ability to synthesize antibody of *any* specificity. This possibility was tested next.

(*d*) Immunization with 100 μg bovine serum albumin (BSA) in Freund's adjuvant and 100 μg in saline.

Animals were injected in the same manner and on the same time scale as

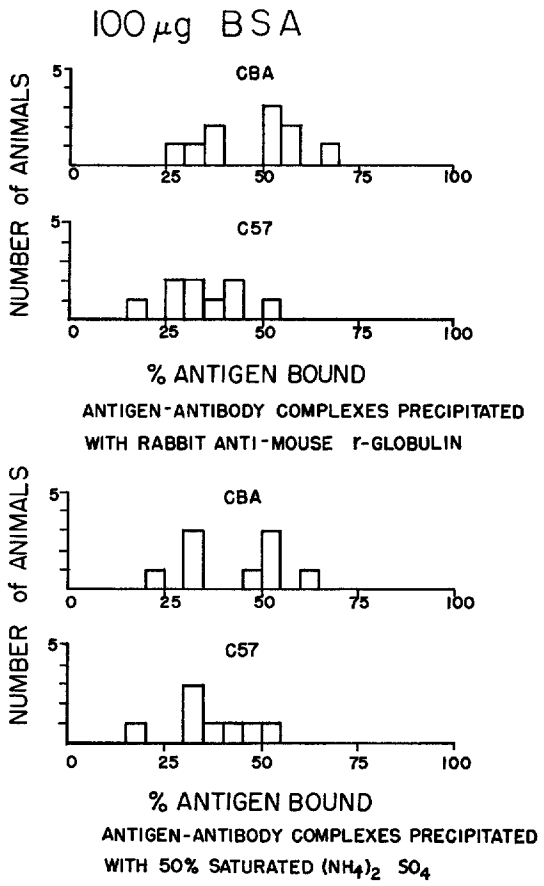

FIG. 6. Immune response of mice given 100 μg bovine serum albumin (BSA) in complete Freund's adjuvant and boosted with 100 μg of the same antigen in saline. In this series, all titrations were done with 20 μg BSA-I^{131} per 10 μl antiserum, and the two strains bind approximately 800 μg BSA/ml antiserum.

for the experiments already described. The results are shown in Fig. 6. In both strains of mice, the response to BSA shows a greater variability than the response to (T,G)-A--L, but the scatter and the averages for the two strains are remarkably similar. In addition, it should be noted that the results are virtually unchanged by the use of 50 per cent saturated $(NH_4)_2SO_4$ to precipitate the BSA–anti-BSA complexes, in place of rabbit anti-mouse γ-globulin. This finding constitutes further evidence that the rabbit anti-mouse γ-globulin

used throughout this study was not selectively precipitating only a particular fraction of the total mouse-γ-globulin present in each antiserum.

On the evidence presented up to this point, it seems reasonable to assume that the quantitative difference in the amount of anti-(T,G)-A--L binding capacity produced in CBA and C57 mice is under genetic control. The nature of this genetic control is of course unknown, but the first step in analyzing it must be an estimate of the number of genetic factors differing between the two strains. An attempt to do this was made by testing larger numbers of the two back-cross strains. On the basis of the previous experiments, a per cent antigen bound value of 0 to 30 per cent was arbitrarily designated the CBA phenotype, a per cent antigen bound value of 30 to 70 per cent was designated the F_1 phenotype, and a value of 70 to 100 per cent was designated the C57 phenotype. If the observed differences were the result of a single genetic factor, then in a large number of $F_1 \times$ CBA mice, half should have the F_1 phenotype, half the CBA phenotype and none the C57 phenotype. The $F_1 \times$ C57 mice should give a similar bimodal distribution. The results of testing 48 $F_1 \times$ CBA mice and 54 $F_1 \times$ C57 mice with 10 μg (T,G)-A--L 509 are shown in Figs. 7 and 8. In both back-cross combinations, *almost* all the mice fall in one of the two phenotype ranges. (The lack of a clear bimodal distribution could be a reflection of imprecision in the assay method and of the slight overlap between the F_1 and parental strains.) However, in both back-cross strains, 4 mice fall outside the predicted limits; *i.e.*, 4 members of the $F_1 \times$ CBA strain fall in the C57 range, and 4 members of the $F_1 \times$ C57 strain fall in the CBA range. In addition, the $F_1 \times$ C57 strain deviates considerably from a fifty-fifty distribution. Although this could conceivably be attributed to statistical variation, this degree of variation was not encountered in 83 CBA, 89 C57, or over 100 F_1 mice. While the predominantly bimodal distribution is compatible with a single genetic factor, the presence of a few animals outside the predicted limits makes equally reasonable the tentative assumption that this genetic difference involves one major factor and an unknown number of modifying factors whose quantitative effect is slight. A choice between this alternative and that of a single genetic factor is not possible on the basis of the evidence to date.

For reasons which will be discussed more fully below, it appears very likely that the antigenic determinants on (T,G)-A--L are restricted to the tyrosine and glutamic acid residues which make up the amino-terminal tips of the multiple side chains of the molecule. This makes it possible to test whether the genetic difference in response to (T,G)-A--L in CBA and C57 mice is a function of the type of antigenic determinant on the molecule, or a function of the A--L "carrier" portion of the (T,G)-A--L. CBA and C57 mice were immunized with either 10 μg or 100 μg of poly(his,glu)-poly DL-ala--polylys, (H,G)-A--L 220, emulsified in complete Freund's adjuvant, and boosted with a similar dose of the antigen in saline. The route and time scale of immunization and bleeding were identical to those used with (T,G)-A--L 509. Antisera

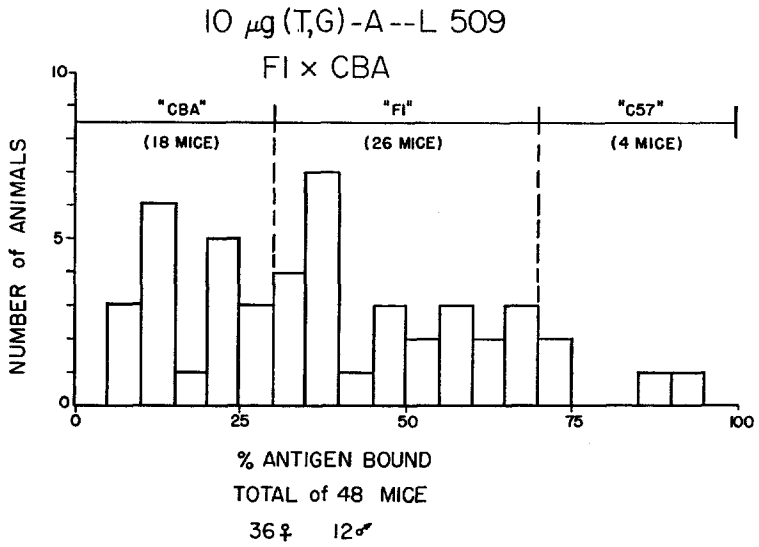

FIG. 7. Immune response of F_1 × CBA mice given 10 μg (T,G)-A--L 509 in complete Freund's adjuvant, and boosted with 10 μg of the same antigen in saline.

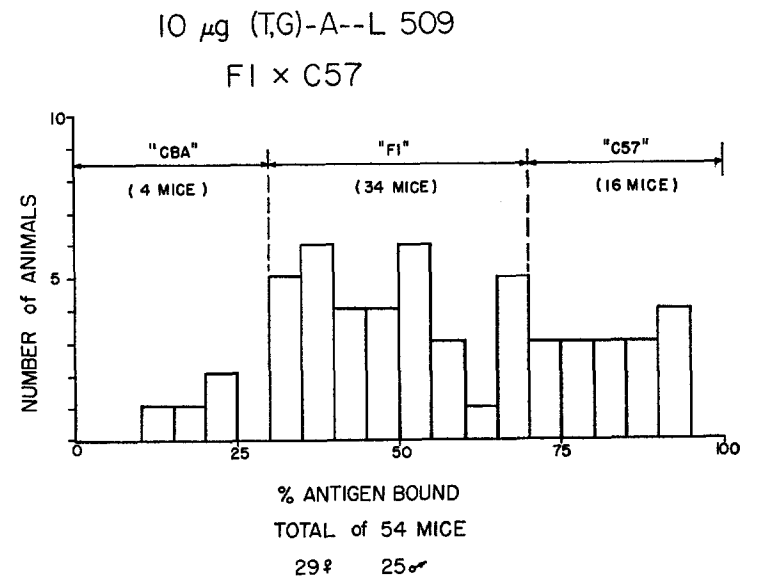

FIG. 8. Immune response of F_1 × C57 mice given 10 μg (T,G)-A--L 509 in complete Freund's adjuvant, and boosted with 10 μg of the same antigen in saline.

were titered with 0.2 μg (H,G)-A--L 220-I^{131} per 10 μl antiserum, to compensate for the fact that the molecular weight of (H,G)-A--L 220 is 40,000, *versus* 230,000 for (T,G)-A--L 509. The results of a typical experiment are seen in Fig. 9. At both dosage levels, CBA mice quite uniformly respond to (H,G)-A--L, while C57 mice give no detectable response. Thus, putting histidine in place of tyrosine in the (T,G)-A--L molecule completely reverses the usual quantitative difference in response to (T,G)-A--L seen in CBA's and C57's. From this fact, it appears that the genetic difference between CBA and C57 mice in their response to (T,G)-A--L is specific for the (T,G) antigenic determinant.

DISCUSSION

Genetic factors of the sort described here have not usually been found even when sought (7). Often, the finding is that noted by Burnet (17) and also found in this study when BSA was the antigen used, namely, a scatter in antibody titer in inbred strains. However, Ipsen (6) has shown that inbred mouse strains differ by as much as tenfold in the amount of tetanus toxoid required to elicit an equal degree of immunity to challenge with tetanus toxin. In addition, Dineen (13) has shown that, in immunizing different inbred strains of mice with sheep red blood cells, the variation between the strains is considerably greater than the variation within a strain, suggesting that the interstrain differences were of genetic origin. Studies of this type were carried further by Stern, Brown, and Davidsohn (5), who demonstrated a genetic difference in the ability of C3H and C57BL mice to produce natural agglutinins for sheep erythrocytes. These authors studied the corresponding F_1 and back-cross strains, with results similar to those presented in this study. Marked differences in response to pneumococcal polysaccharide in five strains of inbred mice were also found by Fink and Quinn (4). Finally, Arquilla and Finn (8) have presented detailed evidence using inbred guinea pigs that antibodies to different antigenic determinants on the insulin molecule are under genetic control. These results, taken with those of Levine *et al.* (9–12), lend support to the idea that the process of antibody formation is under direct genetic control.

One possible reason for the lack of interstrain differences and the scatter in response to complex antigens such as BSA may lie in their complexity, the response to BSA being the sum of several separate responses to all the various antigenic determinants on the BSA molecule (see also reference 7). (T,G)-A--L, on the other hand, is a molecule of restricted antigenic specificity. Branched, multi-poly-DL-alanyl–poly-L-lysine (A--L) is non-antigenic in rabbits (18), and branched multi-(poly-L-glutamyl)-poly-DL-alanyl–poly-L-lysine, (G-A--L), (*i.e.* (T,G)-A--L without the tyrosine) is non-antigenic in rabbits (18) and virtually non-antigenic in mice (19). Fuchs and Sela (20) have shown in rabbits that tyrosine is a prominent part of the antigenic site in (T,G)-A--L.

These authors found that even a large excess of mutli-(poly-L-glutamyl)-poly-DL-alanyl-poly-L-lysine, (G-A--L), did not inhibit the reaction between (T,G)-A--L, and rabbit anti-(T,G)-A--L, whereas several linear or branched polymers containing both tyrosine and glutamic acid were extremely effective inhibitors. Thus, it is probable that in mice as well as in rabbits, (T,G)-A--L is an antigen of restricted specificity, the antigenic sites all being some com-

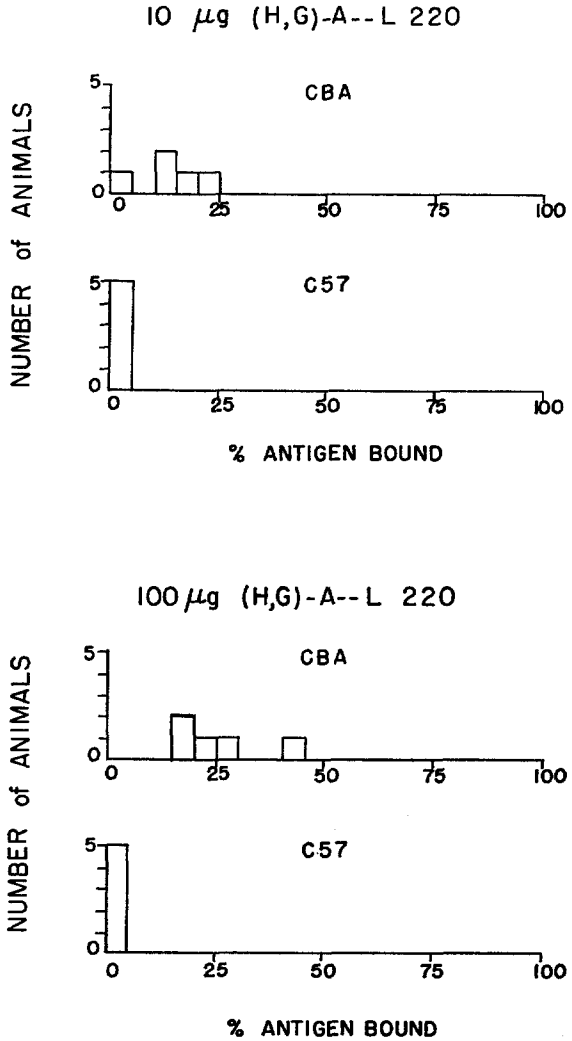

FIG. 9. Immune response of mice given 10 μg or 100 μg (H,G)-A--L 220 in complete Freund's adjuvant, and boosted with 10 μg or 100 μg of the same antigen in saline. In this series, all titrations were done with 0.2 μg (H,G)-A--L I^{131} per 10 μl of antiserum to compensate for the relatively low molecular weight (40,000) of this antigen.

bination of tyrosine, glutamic acid, and, to a much smaller extent, alanine. A second factor permitting demonstration of a strain difference in response may lie in the use of a maximal antigenic stimulus.

The fact that the antigenic sites on (T,G)-A--L are some combination of tyrosine and glutamic acid makes even more striking the demonstration that (H,G)-A--L 220 is non-antigenic in C57 mice and antigenic in CBA mice, a result almost opposite to that obtained with (T,G)-A--L. These results are summarized in Fig. 10. Thus, substituting histidine for tyrosine in the antigenic site of two molecules of the same general pattern of construction completely alters an immune response which has been shown to be genetically determined. The exact nature of this genetic control is unknown, but it ap-

	MOUSE STRAIN	
ANTIGEN	CBA	C57
(T,G)-A--L	10 - 30%	70 - 100%
(H,G)-A--L	10 - 40%	0

FIG. 10. A summary of the immune response of CBA and C57 mice to two related synthetic polypeptide antigens. The animals were immunized and titered in the same way and the results are expressed as "per cent antigen bound".

pears to be due to the nature of the antigenic determinant rather than some other portion of the molecule.

We are dealing then, with the genetic control of the production of antibody against a relatively well defined antigenic site, or sites. A precise understanding of the mechanism underlying this genetic difference might be of great help in understanding mechanisms of antibody formation. At the present time, it is only possible to draw broad inferences, primarily as a guide to further experiments.

It seems unlikely that we are dealing with a genetic factor (or factors) which exerts its effect prior to the actual induction of antibody formation, (such as an enzyme which degrades the antigen in some specific way) since both CBA and C57 mice are capable of responding to (T,G)-A--L. However, it is possible that we are in reality studying two separate antibody responses, one of them present in both strains, the other totally lacking in the CBA's and present in the C57's, where it is produced in much higher titer than the shared response. This possibility is susceptible to experimental test.

It also seems unlikely that the poor response of CBA mice can be explained on the basis of an antigenic determinant found on (T,G)-A--L and also present in the "self" antigens of the CBA strain, thus rendering CBA's tolerant to

this determinant (see reference (21)). Were this the case, the CBA × C57 F_1 mice should also possess this determinant, and respond in a manner similar to the CBA parental strain.

It should be pointed out here that the titering system used in these experiments only reflects antigen-binding capacity, and cannot measure actual amounts of antibody protein present. (Typical precipitin curves have, however, been obtained using pooled CBA × C57 F_1 sera.) It is possible that, for example, CBA mice manufacture as much antibody protein as C57 mice, but with a much lower energy of binding for (T,G)-A--L. While our titering system is ill-adapted to testing this idea, it should be possible to do so using small peptides of tyrosine and glutamic acid. Also, a careful comparison of CBA anti-(T,G)-A--L, CBA anti-(H,G)-A--L, and C57 anti-(T,G)-A--L, with respect to their structure and to their relative abilities to bind either antigen or both antigens together, might reveal similarities or differences between these three antibody populations, and perhaps shed some light on why these two antigens elicit such different patterns of response in the two strains of mice.

Finally, the intermediate response of the CBA × C57 F_1 mice is suggestive of a gene-dose effect in the heterozygote, and might lead one to infer the presence of a structural difference in some as yet unidentified protein, a protein in some way responsible for the recognition of the antigen, or the antibody itself.

Note Added in Proof.—Since this paper was prepared for publication, the findings of Pinchuck and Maurer have become available (*Fed. Proc.*, 1965, **24**, 184). These authors have shown that the ability of mice to respond to a linear, synthetic polypeptide, poly (glu_{57}, lys_{33}, ala_5), appears to be inherited as a simple Mendelian dominant. These results, which are similar to those of Levine *et al.* (9–12) provide additional evidence that the immune response is under direct genetic control.

SUMMARY

Immunization of CBA and C57 mice with a branched, multichain synthetic polypeptide, poly (tyr,glu)-poly DL-ala--poly lys, ((T,G)-A--L), in Freund's complete adjuvant results in a tenfold or more difference in the antigen-binding capacity of sera from the two strains, although they respond equally to bovine serum albumin. Immunization of CBA × C57 F_1, F_1 × CBA, and F_1 × C57 mice reveals definite genetic control of the response to (T,G)-A--L, which appears to be due to a single major genetic factor, with perhaps one or more modifying factors. Immunization of CBA and C57 mice with (H,G)-A--L, a synthetic polypeptide in which histidine replaces tyrosine, gives the opposite result, CBA's respond and C57's do not. From this, it appears that the genetic control of the response to (T,G)-A--L is specific for the antigenic determinant. The implications of these results are discussed.

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