SPECIFIC IMMUNE RESPONSE GENES OF THE GUINEA PIG

I. DOMINANT GENETIC CONTROL OF IMMUNE RESPONSIVENESS TO COPOLYMERS OF L-GLUTAMIC ACID AND L-ALANINE AND L-GLUTAMIC ACID AND L-TYROSINE*

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The demonstration of direct genetic control of specific immune responses followed the introduction of synthetic polypeptides as experimental antigens (1-4). The use of synthetic antigens, whose complexity can be controlled by varying the number and relative proportions of their constituent amino acids (5), has in fact led to several demonstrations of unigenic control of specific immune responsiveness (6). Thus, the ability of guinea pigs to mount immune responses to poly-L-lysine (PLL) 1 and hapten conjugates of this polymer is determined by a single autosomal dominant gene, the “PLL gene” (1, 7, 8). In mice the immune response to GLA5, a random linear polymer of L-glutamic acid and L-lysine containing 5% L-alanine, is under the control of a dominant gene (2). Also in mice, the immunogenicity of three branched synthetic polypeptides containing random sequences of L-glutamic acid with either L-tyrosine, L-histidine, or L-phenylalanine on a backbone of DL-alanine and L-lysine is largely determined by three single dominant genes forming the Ir-I locus (3, 9, 10). Subsequently, single gene control of immune responses to more complex native antigens has also been observed when limiting immunizing doses are used to decrease the interactions between complex antigens and the immune system (11).

The relative rapidity with which “immune response genes” have been demonstrated after the initial identification of the PLL gene suggests that there may well be many more of these genes yet to be discovered. The identification of several immune response genes within a single species should permit preliminary mapping of these genes.

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Abbreviations used in this paper: GA, poly-α-(L-glutamic acid [60%], L-alanine [40%]); GAT, poly-α-(L-glutamic acid [60%], L-lysine [30%], L-tyrosine [10%]); GLA5, random linear polymer of L-glutamic acid and L-lysine containing 5% L-alanine; GT, poly-α-(L-glutamic acid [50%], L-tyrosine [50%]); PBS, 0.015 M phosphate buffer, pH 7.5, containing 0.15 M NaCl; PLL, poly-γ-L-lysine.
genes. Furthermore, studies on the functional interactions of several immune response genes will, hopefully, result in a clearer understanding of the influence of these genes on immune responses to the more complex naturally occurring antigens. With these objectives in mind, we have examined the immunogenicity in guinea pigs of several synthetic polypeptides. We report here on the nature of the immune responses of several inbred and random-bred strains of guinea pigs to three linear polypeptide antigens: (a) GA, poly-α-(L-glutamic acid [60%], L-alanine [40%]); (b) GT, poly-α-(L-glutamic acid [50%], L-tyrosine [50%]); and (c) GAT, poly-α-(L-glutamic acid [60%], L-alanine [30%], L-tyrosine [10%]).

Our data demonstrate that the ability to develop immune responses to both GA and GT is genetically determined in guinea pigs and inherited as separate autosomal dominant traits. GAT, however, which contains both GA and GT determinants among others, is immunogenic in all guinea pigs tested.

Materials and Methods

Polymers.—GA, mol wt 43,000, was obtained from Pilot Chemicals Inc., division of New England Nuclear Corp., Boston, Mass. GT, mol wt 14,500, was obtained from Miles Laboratories, Inc., Elkhart, Ind. GAT, mol wt 25,000, was a gift of Dr. P. H. Maurer.

Animals.—Inbred strain 2 and strain 13 guinea pigs and the outbred N.I.H. multipurpose guinea pigs were obtained from the Animal Production Unit, National Institutes of Health, Bethesda, Md. Outbred Hartley strain guinea pigs were obtained from Canm Research Institute, Inc., Wayne, N.J., and from the Animal Production Unit, N.I.H. Coulsen strain guinea pigs were a gift from the Agriculture Research Service, U.S. Department of Agriculture. All guinea pigs used in these studies weighed between 250 and 450 g.

Preparation of Rabbit Anti-Guinea Pig Gamma Globulin.—Rabbit anti-guinea pig gamma globulin antisera used as the precipitating reagent in the antibody assay were produced in female New Zealand white rabbits weighing 2.5 kg. Each rabbit received 2.5 mg guinea pig gamma globulin (purified as described previously [12]) emulsified in complete Freund’s adjuvant (Difco Laboratories, Detroit, Mich.) and distributed in the four footpads. The rabbits were subsequently injected every 2 wk with 1.0 mg of the guinea pig gamma globulin emulsified in incomplete adjuvant distributed subcutaneously in the flanks. The rabbits were bled by cardiac puncture at 5, 7, and 9 wk after the initial immunization. The pooled sera contained 4 mg precipitating antibody/ml.

Immunization of Guinea Pigs.—Solutions of the synthetic antigens in 0.015 M phosphate buffer, pH 7.5, containing 0.15 M NaCl (PBS) at the desired concentration were emulsified in an equal volume of complete Freund’s adjuvant containing 0.5 mg/ml Mycobacterium butyricum (Difco Laboratories). Each guinea pig received 0.4 ml of the emulsion distributed equally in the four footpads.

Skin Tests.—3 wk after immunization the guinea pigs were injected intradermally with 50 µg of the immunizing polymer in 0.1 ml normal saline. Animals immunized with adjuvant alone were similarly skin tested to evaluate the degree of nonspecific irritation produced by these peptides. The injection sites were examined after 24 hr. Since nonspecific irritation up to 8 mm in diameter was occasionally observed, erythema and induration greater than 8 mm in diameter was considered a positive skin test, and the intensity of the reactions was graded as follows: +, 8-15 mm greatest diameter; ++, 15-25 mm greatest diameter; ++++, >25 mm greatest diameter.

Antibody Assay.—The guinea pigs were bled from the retro-orbital venous plexus 3 wk after immunization, and the sera thus obtained were assayed for antibody activity against
the immunizing polymer using a modified Farr-type assay (13). Radioactive ligands were made by iodinating GT and GAT polymers with $^{125}$I using the chloramine-T method (14). GAT-$^{125}$I was used to assay for both anti-GAT and anti-GA antibody activity. A mixture of 0.025 ml of the appropriate ligand (5–10 m#) and 0.025 ml of a 1:5 dilution in PBS of the serum to be tested was placed at 4°C. After 30 min, 0.05 ml of the rabbit anti-guinea pig gamma globulin diluted 1:1 with PBS was added. After an additional 30 min at 4°C, the samples were centrifuged at 2500 rpm for 15 min, and 0.05 ml of the resulting supernates were counted in a Packard gamma spectrometer (Packard Instrument Co., Downers Grove, Ill.). The radioactivity remaining in the supernates of the test samples was compared to the radioactivity remaining when normal guinea pig serum was used, and the data expressed as per cent antigen bound according to the formula:

$$\% \text{ binding} = \left( 1 - \frac{\text{cpm in supernate from test sera}}{\text{cpm in supernate from normal sera}} \right) \times 100.$$ 

For both the GT-$^{125}$I and GAT-$^{125}$I ligands used in this study, a comparison of the binding values of a panel of control nonimmune sera indicated that greater than 10% antigen binding was needed to demonstrate the presence of specific antibody in our test antisera. This value is greater than the difference in binding observed between any two nonimmune sera, and is approximately three standard deviations from the mean per cent binding of all nonimmune sera tested.

RESULTS

When immunized with 500 #g of a random copolymer of L-glutamic acid and L-alanine (GA), all inbred strain 2 guinea pigs tested respond with both delayed hypersensitivity and serum antibody to GA (Table I). An adequate immunizing dose of GA is needed to elicit uniform responsiveness, for only 73% of strain 2 guinea pigs respond when immunized with 100 #g of GA with weak delayed skin reactions. Inbred strain 13 guinea pigs, on the other hand, do not respond to immunization with 500 #g of GA. They do not demonstrate delayed skin reactivity and their sera have no detectable anti-GA antibody (Fig. 1).

All F1 hybrid offspring resulting from the mating of strain 2 male with strain 13 female guinea pigs respond to immunization with 500 #g of GA, demonstrating that the ability to respond to GA is inherited as an autosomal dominant trait. Their delayed skin reactions are, as those in the strain 2, of moderate intensity. Their mean serum antigen binding, 31.2%, is somewhat lower than the 41.5% mean antigen binding seen in the sera of strain 2 animals, but this difference is not statistically significant. A variable percentage of random-bred Hartley strain guinea pigs develop delayed-type hypersensitivity to GA (Table I). The response rate to GA immunization depends upon the source of the animals. Thus, in our experiments, 90% of the Hartley guinea pigs obtained from the N.I.H. Animal Production Unit (N.I.H. Hartleys) demonstrate delayed skin reactivity to GA 3 wk after a single immunization with 100 #g of GA. However, under the same conditions, less than 60% of Hartley guinea pigs obtained from Camm Research Institute, Inc. (Camm Hartleys) develop delayed hypersensitivity to GA.
### TABLE I

| Strain       | Immunizing dose | No. of animals | No. of responders* | Intensity of skin reaction | % Antigen bound§ |
|--------------|-----------------|----------------|-------------------|---------------------------|-----------------|
|              | mf              | Mean (S.E.)    | Mean (S.E.)       |                           |                 |
| 2            | 100             | 26             | 19                | ±                         | 21.0 (2.21)     |
| 2            | 500             | 11             | 11                | ++                        | 41.5 (4.00)     |
| 13           | 500             | 10             | 0                 | —                         |                 |
| (2 × 13)F₁  | 500             | 6              | 6                 | ++                        | 31.2 (6.83)     |
| N.I.H.       | 100             | 20             | 18                | +++                       | 64.1 (4.22)     |
| Hartley Camm| 100             | 54             | 31                | +++                       | 49.8 (3.07)     |
| Hartley     | 500             | 20             | 9                 | +++                       | 37.3 (5.15)     |
| Hartley     | 500             | 30             | 1                 | +                         | 27.7 (0.0)      |
| N.I.H.       | 500             | 30             | 1                 | +                         | 27.7 (0.0)      |
| multipurpose |                 |                |                   |                           |                 |

* Responders are those animals exhibiting delayed skin reactivity after an intradermal injection of 50 μg of GA 3 wk after immunization.

† Delayed skin reactions read 24 hr after injection are graded according to the extent of erythema and induration as follows: — = <8 mm greatest diameter; + = 8–15 mm greatest diameter; ++ = 15–25 mm greatest diameter; +++ = >25 mm greatest diameter.

§ The amount of GAT-125I bound by a 1:5 dilution of the test serum compared to the amount bound by a 1:5 dilution of normal serum. The assay is described in detail in Materials and Methods.

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![Graph](image-url)

**Fig. 1.** Antibody response to 0.5 mg GA in individual inbred strain 2 and strain 13 guinea pigs expressed as the per cent binding of GAT-125I, 0.15 μg/ml, by a 1:10 dilution of antiserum (see Materials and Methods).
Among Hartley animals, also, there is an excellent correlation between the ability to respond to GA immunization with delayed hypersensitivity and the ability to produce antibody directed against GA. All sera from "responder" guinea pigs (i.e., those animals demonstrating delayed hypersensitivity to the immunizing antigens) contain anti-GA antibody (Fig. 2). Sera from most "nonresponder" Hartley guinea pigs do not contain any detectable anti-GA antibody. The few that do have very low levels.

A fivefold increase in the immunizing dose of GA does not increase either the response rate or the level of antibody produced in Hartley responders. In fact, increasing the immunizing dose from 100 to 500 μg results in a slight decrease from 58 to 42% responders and a drop in their mean serum antigen binding from 49.8 to 37.3%. Both of these differences, however, are not statistically significant.

In contrast to the rate of GA responsiveness in Hartley animals, the random-bred pigmented N.I.H. multipurpose guinea pigs demonstrate a very low GA response rate. Only one GA responder was found among the 30 animals immunized with 500 μg GA.

When immunized with 500 μg GT, all strain 13 guinea pigs respond with moderately strong delayed-hypersensitivity skin reactions and significant levels of circulating anti-GT antibody (Table II). Inbred strain 2 guinea pigs, on the other hand, do not respond with either delayed hypersensitivity or circulating
antibody after immunization with 500 μg GT (Fig. 3). All F1 hybrid offspring from matings of strain 13 males with strain 2 females respond to immunization with 500 μg GT. Thus, the ability to respond to GT is also inherited as an autosomal dominant trait. The delayed skin reactions to GT observed in the F1 hybrids are, as in strain 13 animals, moderately intense. The anti-GT antibody level in the sera of the F1 hybrids, 19.8% mean antigen binding, is considerably lower than the 38.1% mean antigen binding produced in strain 13 guinea pigs similarly immunized.

| Strain | Immunizing dose | No. of animals | No. of responders* | Intensity of skin reaction‡ | % Antigen bound§  |
|--------|----------------|----------------|-------------------|-----------------------------|------------------|
|        | μg             |                |                   |                             |                  |
| 13     | 100            | 12             | 6                 | ±                           | 14.7 (2.33)      |
| 13     | 500            | 13             | 13                | ++                          | 42.7 (3.79)      |
| 2      | 500            | 10             | 0                 | −                           | 4.6 (1.18)       |
| (13 × 2)F1 | 500 | 6             | 6                 | ++                          | 19.8 (1.51)      |
| N.I.H. Hartley | 100 | 23             | 11                | ++                          | 32.5 (6.97)      |
| Camm Hartley | 100 | 88             | 55                | ++                          | 25.0 (2.25)      |
| Camm Hartley | 500 | 18             | 15                | +++                         | 48.2 (4.33)      |
| N.I.H. multipurpose | 500 | 65             | 56                | +++                         | 66.2 (2.37)      |

* Responders are those animals exhibiting delayed skin reactivity after an intradermal injection of 50 μg of GT 3 wk after immunization.
‡ Delayed skin reactions read 24 hr after injection are graded according to the extent of erythema and induration as described in Table I.
§ The amount of GT-125I bound by a 1:5 dilution of the test serum compared to the amount bound by a 1:5 dilution of normal serum. The assay is described in detail in Materials and Methods.

When the immunizing dose of GT is decreased significantly, the response of inbred strain 13 guinea pigs becomes inconsistent. Only about half of the strain 13 animals immunized with 100 μg of GT demonstrate delayed skin reactivity to GT. In addition, their positive reactions are quite weak and the level of anti-GT antibody produced is very low.

After immunization with 100 μg GT in complete Freund’s adjuvant, from 50–60% of Hartley guinea pigs, depending upon their source, develop delayed-hypersensitivity skin reactions when challenged intradermally with 50 μg GT (Table II). Both Camm and N.I.H. Hartley responder guinea pigs produce relatively low levels of serum anti-GT antibody at this immunizing dose. The mean serum antigen-binding level found in responder N.I.H. Hartleys
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(32.5%) is somewhat higher than that found in Camm Hartleys (25.0%), but this difference is not statistically significant.

There is a correlation between responder status, after immunization with 100 μg GT, and the presence of circulating antibody. The sera from most Camm Hartley nonresponders are devoid of detectable anti-GT antibody, while the sera from most responders contain a significant amount of it. There are, however, a few nonresponders producing detectable anti-GT antibody, albeit at very low levels, and several responder guinea pigs that do not produce detectable antibody.

Increasing the immunizing dose of GT from 100 to 500 μg per animal results in a somewhat higher response rate of 80% and, concomitantly, higher serum antigen-binding levels. The increase from 25% mean serum antigen binding after immunization with 100 μg GT to 48% after immunization with 500 μg GT in Camm Hartleys is statistically significant. At the 500 μg GT immunizing dose there is a good correlation between responder status and the presence of circulating anti-GT antibody (Fig. 4). All responders produce detectable anti-GT antibody. Of the three nonresponders in this group, the serum of one has no detectable anti-GT antibody, while the sera of the other two have very low GT binding levels.

N.I.H. multipurpose guinea pigs have a very high rate of GT responsiveness. About 84% of those immunized with 500 μg GT respond with both delayed hypersensitivity and high levels of anti-GT antibody. The 66.2% mean

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**Fig. 3.** Antibody response to 0.5 mg GT in individual inbred strain 2 and strain 13 guinea pigs expressed as the per cent binding of GT-125I, 0.1 μg/ml, by a 1:10 dilution of antiserum (see Materials and Methods).
serum antigen binding of N.I.H. multipurpose GT responders is considerably
greater than the anti-GT levels produced by any of the other strains tested.

TABLE III

| Strain            | Immunizing dose | No. of animals | No. of responders* | Intensity of skin reaction† | % Antigen bound§ |
|-------------------|-----------------|----------------|-------------------|-----------------------------|-----------------|
|                   | µg              |                |                   |                             |                 |
| 2                 | 100             | 7              | 7                 | ++                          | 72.6 (5.71)     |
|                   | 200             | 5              | 5                 | ++                          | 77.3 (3.00)     |
| 13                | 100             | 10             | 10                | +                            | 49.0 (4.65)     |
| Camm Hartley      | 100             | 19             | 19                | +++                         | 76.5 (2.19)     |
| Coulson           | 100             | 8              | 8                 | ++                          | 72.8 (3.86)     |
| N.I.H. multipurpose| 100             | 26             | 26                | ++                          | 53.9 (3.39)     |

* Responders are those animals exhibiting delayed skin reactivity after an intradermal injection of 50 µg of GAT 3 wk after immunization.
† Delayed skin reactions read 24 hr after injection are graded according to the extent of erythema and induration as described in Table I.
§ The amount of GAT-¹²⁵I bound by a 1:5 dilution of the test serum compared to the amount bound by a 1:5 dilution of normal serum. The assay is described in Materials and Methods.

![Graph](image)

**Fig. 4.** Antibody response to 0.5 mg GT in individual responder (skin test positive) and nonresponder (skin test negative) Camm Hartley guinea pigs expressed as the per cent binding of GT-¹²⁵I, 0.1 µg/ml, by a 1:10 dilution of antisera (see Materials and Methods.)

None of the N.I.H. multipurpose GT nonresponders produced any detectable anti-GT antibody.

After immunization with 100 µg GAT, all guinea pigs respond with both delayed hypersensitivity and circulating anti-GAT antibody (Table III).
Among the 85 guinea pigs from five different strains tested, there is not a single nonresponder to GAT. Between the two inbred strains, strain 2 guinea pigs produce significantly higher anti-GAT antibody levels. Increasing the immunizing dose fivefold did not abolish this difference. Among the different

random-bred strains immunized with GAT, the mean serum antigen-binding levels produced by Hartley and Coulsen guinea pigs are similar to the level found in strain 2 guinea pigs. The N.I.H. multipurpose guinea pigs, however, like strain 13 animals, produce significantly lower levels of anti-GAT antibody.

The results of our studies on the immunogenicity of GA, GT, and GAT are summarized in Fig. 5. Strain 2 guinea pigs all respond to GA and GAT but not to GT. Strain 13 guinea pigs respond to GT and GAT but not to GA. Among the outbred Hartley animals, all respond to GAT, but a variable percentage, depending upon their source, respond to GA and GT.

Fig. 5. Frequency of responsiveness to GA, GT, and GAT in inbred strain 2 and strain 13 and random-bred Hartley strain guinea pigs obtained from two different sources.
DISCUSSION

Inbred strain 2 guinea pigs all respond to immunization with an adequate dose of GA with both cellular and humoral immunity. Inbred strain 13 guinea pigs, on the other hand, are unable to make either cellular or humoral immune responses when similarly immunized. Thus, it appears that the ability to respond immunologically to GA is a genetically determined trait that is expressed as an "all-or-none" phenomenon within the detection limits of our assays.

Our observations that all F1 hybrid progeny, resulting from matings between these two strains, respond to GA immunization in a manner similar to their strain 2 parent demonstrate the dominant character of the genetic trait determining GA responsiveness. This observation proves, furthermore, that we are not dealing with a sex-linked trait, for if it were, the male offspring of matings between strain 2 males and strain 13 females would be nonresponders. Hence, the ability to respond immunologically to GA is inherited as an autosomal dominant trait.

The pattern of GA responsiveness among the several guinea pig strains closely parallels the pattern of PLL responsiveness (8). Both PLL and GA are immunogenic in all strain 2 and some Hartley guinea pigs but not in any strain 13 animals. It has already been shown, however, that immune responsiveness to GA is not controlled by the PLL gene (15). As we demonstrate in greater detail in the subsequent paper (16), GA and PLL responsiveness are dissociated in some Hartley guinea pigs. There are, therefore, separate genes governing the ability to develop immune response to these two polymers.

In contrast to the pattern of GA responsiveness among guinea pig strains, all strain 13 guinea pigs respond with both cellular and humoral immunity after immunization with GT in complete Freund's adjuvant, while strain 2 animals are completely unresponsive. As is the case with GA responsiveness, however, all the F1 hybrid progeny from matings of these two strains are GT responders, thus demonstrating that immune responsiveness to GT is also inherited as an autosomal dominant trait. Furthermore, in studies to be reported of the responsiveness of the backcross progeny of (2 X 13)F1 animals mated to the appropriate nonresponder parental strain, it will be shown that separate single autosomal dominant genes control responsiveness to GA and GT respectively in strains 2 and 13.

The ability to mount an immune response directed against GAT does not appear to be under such simple genetic control. Unlike GA or GT, GAT is immunogenic in all guinea pigs tested. Some characteristics of the immune response to GAT, however, do seem to be genetically influenced. In fact,
both strain 13 and N.I.H. multipurpose guinea pigs produce distinctly lower amounts of anti-GAT antibody than do the other strains. These two strains have in common a very high rate of GT responsiveness and, perhaps more importantly, a very low percentage of GA responders. Structurally, GAT is GA with 10% of its alanine residues replaced with tyrosine groups. The decreased antibody levels in the strain 13 and N.I.H. multipurpose guinea pigs may be due in part to their inability to recognize or respond to the GA determinants which must comprise a large part of the GAT molecule. Evidence to support this hypothesis will be presented subsequently in a more detailed study of the genetically determined variations in immune responses to GAT.3

The immunogenicity of GT, GA, and GAT in inbred and Hartley guinea pigs has been reported on previously by other workers (17, 18). Ben-Efraim et al. reported that GT was immunogenic in strain 13 but not strain 2 guinea pigs (17). However, they failed to observe responsiveness to GT in (2 × 13)F1 animals. This was probably the result of immunizing with only 100 μg of GT which, as we demonstrate in this study, is a submaximal immunizing dose.

Ben-Efraim and Maurer (18) have reported on the immunogenicity of GA and GAT in strain 2, strain 13, and Hartley guinea pigs using the same polymers we have used. Their results are consistent with ours with the exception that they did not observe uniform responsiveness to GA in strain 2 animals, perhaps because they immunized with 100 μg of the antigen which, as we have shown in this study, does not elicit maximal responses. The lack of uniform delayed-hypersensitivity responses to GAT in both of their studies may be due to the use, by these workers, of adjuvant containing Mycobacterium tuberculosis (H37Rv) at 10 mg/ml. In certain situations this adjuvant has been shown to suppress immune responses to synthetic polypeptide antigens (19). We have noted this phenomenon with both GT and GAT.4

We have demonstrated in this study the existence of two distinct genetic systems controlling immune responsiveness of guinea pigs to two different synthetic polypeptide antigens, GA and GT. Since like the PLL gene these are dominant genetic traits, the lack of responsiveness in nonresponders cannot be attributed to tolerance, that is, a cross-reactivity of the synthetic antigen with a component of the nonresponding guinea pig. The data reported in this paper and also that to be presented demonstrate that the GA gene and the GT gene behave as unique autosomal dominant specific immune response genes similar in their properties to the PLL gene.

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SUMMARY

The immunogenicity of three random copolymers of amino acids with L-
glutamic acid and L-alanine (GA), L-glutamic acid and L-tyrosine (GT), or
L-glutamic acid, L-alanine, and L-tyrosine (GAT), administered in complete
Freund's adjuvant, was studied in several inbred and random-bred guinea
pig strains. The animals were tested for delayed sensitivity and their sera were
assayed for the presence of antibody directed against the immunizing polymer.
All of the guinea pigs developing delayed hypersensitivity also had significant
antibody levels in their sera.

Inbred strain 2 guinea pigs responded to immunization with GA, but failed
to form detectable responses to GT. Inbred strain 13 animals, on the other
hand, responded to GT, but not to GA. The (2 × 13)F₁ hybrids responded to
both GA and GT with both delayed hypersensitivity and circulating antibody.
Thus, the ability of these inbred guinea pigs to respond immunologically to
GA or GT is controlled by distinct autosomal dominant genes.

A variable percentage of random-bred guinea pigs, depending on their
source as well as their strain, responded to immunization with GA and
with GT.

All guinea pigs, both inbred and random bred, responded to immunization
with GAT. The ability to respond immunologically to GAT, therefore, does
not seem to be under simple genetic control. However, the levels of anti-GAT
antibody found in the sera of animals lacking the ability to respond to GA
were much lower than those detected in GA responder animals.

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