GENETIC CONTROL OF THE ANTIBODY RESPONSE IN INBRED MICE

Transfer of Response by Spleen Cells and Linkage to the Major Histocompatibility (H-2) Locus*

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The antibody responses of CBA and C57 mice to a series of multichain synthetic polypeptide antigens are quantitative traits which are under a dominant, determinant-specific type of genetic control (1, 2). These antigens (2) are composed of a polylysine backbone with side chains of poly-DL-alanine terminating in short, random sequences of either tyrosine and glutamic acid, [(T, G)-A--L], or histidine and glutamic acid, [(H, G)-A--L], or phenylalanine and glutamic acid, [(P, G)-A--L]. The multipoly-alanyl--polylysine (A--L) part of these antigens is not antigenic by itself, and the antibody response to (T, G)-A--L is specific for the tyrosine, glutamic acid, and alanine at the end of each side chain (1). CBA and C3H mice respond well to (H, G)-A--L and poorly to (T, G)-A--L, while C57 mice respond poorly to (H, G)-A--L and well to (T, G)-A--L (1, 2). The quantitative difference between CBA and C57 mice with respect to anti-(T, G)-A--L response is approximately tenfold (1). While these differences are clearly quantitative, high responder mice will be termed responders, and low responder mice will be termed nonresponders for the sake of simplicity.

The mechanism of the genetic control responsible for these differences in immune response is unknown. It has been shown that the ability to respond to (T,G)-A--L is not correlated with the immunoglobulin class of the resultant anti-(T,G)-A--L or with the possession by the responding animal of the immunoglobulin allotype of the high responder parental strain (3). No evidence is yet available with regard to possible genetic control of the structure of the anti-(T,G)-A--L combining site itself. In the present study, attempts were made to transfer the ability to respond to (T,G)-A--L by the transfer of

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spleen cells from responder to nonresponder animals in order to find out whether this genetic control is directly related to the process of antibody formation, or is an indirect result of genetic effects on processes completely unrelated to antibody formation. Examples of effects on processes unrelated to antibody formation might include the production (by the nonresponder strain) of substances which bind the antigen, or enzymes which destroy it. The results indicate that this genetic control is directly related to the process of antibody formation and can be localized to spleen cells, since it is possible to transfer the ability to respond well to (T,G)-A--L from high responder (C3H × C57Bl/6) F_1 mice into irradiated, low responder C3H recipients by transferring dissociated F_1 spleen cells. In the course of these studies, a search for a more convenient cell transfer system utilizing congenic strains of mice led to the discovery that the major genetic factor controlling the ability to respond well to (T,G)-A--L and (H,G)-A--L is genetically linked to the *Histocompatibility-2* (*H-2*) locus and can thus be localized to the IXth mouse linkage group.

Materials and Methods

C3H and C57Bl/6 mice were obtained from the Jackson Memorial Laboratories, Bar Harbor, Maine. The (C3H × C57Bl/6) F_1 mice were bred and maintained at the U.S. Naval Radiological Defense Laboratory, San Francisco.

CBA, C57, (CBA × C57) F_1, F_1 × CBA, and F_1 × C57 mice were bred from strains originally obtained from the National Institute for Medical Research, Mill Hill, London, and were maintained at Stanford. Mice of the following strains were purchased from the Jackson Memorial Laboratories: A, A.BY, C57Bl/10, B10.BR, C3H/He, and C3H.SW.

The antigens used, and the methods of immunization and bleeding have been described previously (1, 2). In general, mice were given a primary stimulus with 10 μg (T,G)-A--L 509 (1) in complete Freund's adjuvant in the hind footpads or subcutaneously in the abdominal wall. The adjuvant contained 2 mg/ml killed *Mycobactericum tuberculosis* H37/ra, kindly supplied by Dr. Sidney Raffel. 3 wk after the primary stimulus, the mice received 10 μg (T,G)-A--L 509 in aqueous solution as a secondary stimulus. The animals were bled 10 days after the secondary stimulus. Any deviations from this procedure will be indicated.

The antibody assay used was a modification of that previously described (1), based on the method described by Herzenberg et al. (4). 50 μl of antigen solution, containing 0.0025 μg of (T,G)-A--L 509 ^{125}I and 1% bovine serum albumin in phosphate buffered saline (0.15 N, pH 7.0) was mixed with 25 μl of the appropriate dilution of mouse antiserum ($\frac{1}{10}$ through $\frac{1}{500}$) and incubated 30 min at 37°C and 2 hr at 4°C. After incubation, 25 μl of the appropriate dilution of polyvalent rabbit anti-mouse γ-globulin antiserum (undiluted, $\frac{1}{2}$, $\frac{1}{5}$, $\frac{1}{10}$) was added, followed by a second period of incubation. The mixture was then centrifuged at 10,000 g at 4°C in a Servall GSA rotor. At this speed, the very small amounts of soluble complexes composed of (T,G)-A--L, mouse anti-(T,G)-A--L, and rabbit anti-mouse γ-globulin are sedimented on the bottom of the centrifuge tube, and one-half (50 μl) of the supernatant can then be removed and counted in a well-type gamma scintillation counter and the results expressed as the per cent antigen bound in the complexes. Antibody titers are thus given as per cent values. Extensive preliminary testing showed that (a) the per cent antigen bound at all dilutions of normal mouse serum was 0–5%, (b) 86–90% of the counts were precipitable by specific antibody, (c) the relative values for per cent antigen bound were very similar to those obtained by the method previously used (1). If anything, differences between CBA

and C57 sera are larger at high dilutions of antisera. Antisera were frequently tested at more than one dilution, and the same dilution was tested against more than one dilution of rabbit anti-mouse γ-globulin to eliminate possible artifacts produced by using too high a dilution of antiserum, by using too little rabbit anti-mouse γ-globulin to precipitate all the mouse antibody, or by using such an excess of rabbit antibody that anti-(T,G)-A--L sites on mouse antibody were sterically blocked.

12–16 wk old thymectomized and nonoperated male and female C3H and (C3H × C57Bl/6) F_1 mice were used as recipients in cell transfer studies. All mice received 840 rad whole body X-radiation (250 kvp, 15 ma; half-value layer, 1.5 mm Cu; 30 rad/min). The mice were housed 10 to a cage. Adult male and female C3H and (C3H × C57Bl/6) F_1 mice were used as donors of spleen and lymph node cells. The spleen and lymph nodes were removed aseptically and the cells were gently dissociated in cold TC 199 (Difco Laboratories, Inc., Detroit, Mich.). Aliquots of each cell suspension, containing between 100×10^6 and 150×10^6 viable nucleated cells, were injected intraperitoneally into the hosts shortly after they were irradiated.

H-2 typing was done by standard methods of hemagglutination (5), using anti-H-2^b and anti-H-2^k antisera kindly supplied by Dr. L. A. Herzenberg. The results were scored without knowledge of the source of red cells, and with the presence in random order of known positive and negative red cell suspensions. Great difficulty was encountered in correctly detecting H-2^b on C57 and (CBA × C57) F_1 red cells. For this reason, three separate determinations were done on all (CBA × C57) F_1 × CBA mice tested, and the controls used were C3H (H-2^k) and C3H.SW (H-2^b) red cell suspensions, which gave accurate and reproducible results.

RESULTS

Adoptive Transfer of a Secondary Antibody Response to (T,G)-A--L.—Table I presents the results obtained by transferring spleen cells from animals already immunized to (T,G)-A--L into irradiated C3H recipients. Groups 1 and 2 are the controls and show the response of intact (C3H × C57Bl/6) F_1 mice and C3H mice to a standard immunization with (T,G)-A--L. The F_1 mice have a per cent antigen bound value of 53% and C3H mice have a per cent antigen bound value of 3%. Titering these antisera at lower dilutions does not appreciably change the results. Groups 3, 4, and 5 show the results of transferring normal or immunized C3H spleen cells into irradiated C3H recipients followed by a second injection of antigen either at the time of transfer (group 4) or 10 days after transfer (group 5). The results in all three cases indicate that these animals behave in the same way as the low responder intact C3H mice. Groups 6, 7, and 8 show the results of transferring normal or immunized (C3H × C57Bl/6) F_1 spleen cells into irradiated C3H recipients followed by a second injection of antigen either at the time of transfer (group 7) or 10 days after transfer (group 8). Normal F_1 spleen cells transferred into irradiated C3H recipients followed by a standard injection of (T,G)-A--L in aqueous solution failed to give any response. Immunized F_1 spleen cells transferred into irradiated C3H recipients followed by a secondary stimulus either at the time of transfer or 10 days after transfer gave an antibody response 10 days later which is almost as high as that given by the intact (C3H × C57Bl/6)

TABLE I

*Adoptive Transfer of a Secondary Response to (T, G)-A--L with
(C3H × C57Bl/6) F$_1$ Spleen Cells*

Recipients*	Recipient irradiation prior to transfer	Cell donor‡	Time of secondary stimulus§	Average titer prior to secondary stimulus‖	Average titer and range 10 days after secondary stimulus‖
	rad				%
1. (C3H × C57Bl/6) F$_1$ (10)	None	None	3 wk	—¶	53 (18–68) (1/500) 71 (46–81) (1/100) 70 (44–81) (1/10)
2. C3H(10)	None	None	3 wk	—	1 (0–2) (1/500) 1 (0–3) (1/100) 1 (0–2) (1/10)
3. C3H(8)	840	Untreated C3H	Time of transfer	—	4 (0–9) (1/10)
4. C3H(9)	840	C3H immunized § 3 wk prior to transfer	Time of transfer	—	8 (0–15) (1/10)
5. C3H(10)	840	Same as 4	10 days after transfer	2% (1/50) 2% (1/10)	4 (0–20) (1/50)
6. C3H(10)	840	(C3H × C57Bl/6) F$_1$ untreated	Time of transfer	—	0 (1/50)
7. C3H(10)	840	(C3H × C57Bl/6) F$_1$ immunized 3 wk prior to transfer	Time of transfer	—	59 (1 = 2%; 9 = 22–81%) (1/50)
8. C3H(10)	840	Same as 7	10 days after transfer	5% (1/50) one mouse had titer = 32%	56 (32–79) (1/50)

* Number of recipients in parentheses; approximately equal numbers of males and females.

‡ *All* irradiated hosts received 3 × 10⁶ normal C3H bone marrow cells i.v., as well as 125–150 × 10⁶ spleen cells i.p., from the indicated cell donors.

§ Primary stimulus in either intact (groups 1 and 2) animals or in the spleen cell *donors* (4, 5, 7, 8) was 10 μg (T, G)-A--L 509 in complete Freund's adjuvant. Secondary stimulus in all groups was 10 μg (T, G)-A--L in aqueous solution.

‖ Fractions in parentheses indicate the dilutions at which the sera were titered. (Please see Materials and Methods.)

¶ —, indicates that sera were not obtained prior to the secondary stimulus.

F_1 mice in group 1. That this is definitely a secondary response is shown by group 8 in which the titer, 10 days after transfer and just prior to boosting, is 5%, rising to 56% 10 days after the booster injection. Groups 3 and 6 were later given a standard primary and secondary stimulus with (T,G)-A--L. Both groups failed to respond, although on the basis of the above results, group 6 was expected to respond. No definite explanation can be given for this anomalous result. All the animals in group 6 accepted F_1 skin grafts and their sera contained immunoglobulin of donor allotype. It is possible that a

TABLE II

*Adoptive Transfer of a Primary Response to (T, G)-A--L to Lethally Irradiated C3H Hosts with $(C3H \times C57Bl/6)$ F_1 Spleen Cells**

Recipients‡	Primary stimulus,§ days after radiation	Titer 10 days after secondary stimulus||
Thymectomized C3H ♀ (7)	0	52, 19, 10, 58, 0, 0, 65 (1/50)
Normal C3H ♀ (7)	0	13, 60, 41, 51, 50, 8, 54 (1/50)
Normal C3H ♂ (9)	21	39, 8, 49, 45, 0, 63, 52, 50, 73 (1/50)
Thymectomized C3H ♀ (5)	21	46, 17, 54, 72, 26 (1/50)
Normal (C3H × C57Bl/6) F_1 ♀ (5)	21	76, 59, 58, 50, 47 (1/50)
Normal (C3H × C57Bl/6) F_1 ♀ (4)	0	69, 76, 71, 65 (1/50)

Summary: $F_1 \rightarrow$ C3H = 38% (19/28)
$F_1 \rightarrow F_1$ = 63% (9/9)
C3H → C3H = 8% (see Table I)

* Spleen cell dose in all these transfers was 100–150 × 10⁶ (C3H × C57Bl/6) F_1 spleen cells per recipient.

‡ Number of recipients in parentheses.

§ 1° stimulus was 10 μg (T, G)-A--L 509 in complete Freund's adjuvant. 2° stimulus was 10 μg (T, G)-A--L 509 in aqueous solution given 3 wk later.

|| Titer is average per cent antigen bound. Figures in parentheses indicate dilution at which antisera were titered.

first injection of (T,G)-A--L in aqueous solution induced partial or complete tolerance in the F_1 spleen cells.

These results show that it is possible to transfer a secondary response to (T,G)-A--L by transferring previously immunized spleen cells from an F_1 responder strain into an irradiated, nonresponder parental strain.

Adoptive Transfer of a Primary Antibody Response to (T,G)-A--L.—The results of transferring normal F_1 spleen cells into irradiated F_1 or C3H recipients, followed by immunization with (T,G)-A--L in Freund's adjuvant and a secondary stimulus of (T,G)-A--L in aqueous solution, are shown in Table II. This table presents the results of several different experiments which can-

not easily be grouped together because the recipients in some cases were thymectomized. The results show that in 19 out of 28 cases in which normal F_1 spleen cells were transferred into irradiated C3H recipients (thymectomized [7/12] or nonthymectomized [12/16]) followed by immunization either immediately after cell transfer or 3 wk after cell transfer, the recipients gave an antibody response with an average per cent antigen bound value for the entire group of 38%. The average per cent antigen-bound value for the 19 animals in which transfer was successful is 53%. Both these values are considerably in excess of the immune response of intact C3H mice to immunization with (T,G)-A--L (see Table I). When F_1 spleen cells were transferred into irradiated F_1 recipients, and the recipients immunized either at the time of transfer or 3 wk after transfer, all of the animals responded with an antibody response that gave an average per cent antigen-bound value of 63%. This is as high as the response of intact F_1 mice to this type of immunization. The transfer of F_1 spleen cells into C3H mice transfers the ability to respond almost as well as intact F_1 mice (antibody titers in the two groups are completely overlapping) and much better than intact C3H mice. It should be noted that the F_1 spleen cells remain capable of responding up to 3 wk after transfer, and that they respond equally well in thymectomized or nonthymectomized C3H hosts. The fact that F_1 cells appear not to respond quite as well or as reproducibly in C3H hosts as they do in syngeneic F_1 hosts appears reasonable since the F_1 cells may not thrive as well in a parental strain as in a syngeneic host (6).

From these results, it seems clear that there is no property of the C3H host which renders it incapable of supporting a response to (T,G)-A--L, but rather that the C3H spleen cells are incapable of responding to (T,G)-A--L, while F_1 spleen cells are capable of responding to this antigen in either a C3H or an F_1 host.

Immune Response of Congenic Strains of Mice to (T,G)-A--L *and* (H,G)-A--L.—Numerous attempts to transfer spleen cells from C57 donors to CBA or C3H recipients led to failures attributable to radiation deaths and to deaths due to graft vs. host disease. Because of these difficulties, a systematic study of several congenic strains of mice available from the Jackson Memorial Laboratories was undertaken in an attempt to find strains of mice which were congenic with either C57 or C3H, but which contained the major histocompatibility antigens (*H-2*) of either the C3H or C57 strains, respectively. By this means, it was hoped to develop a more convenient cell transfer system in which graft vs. host disease would be minimized. The results of these studies are shown in Table III. C3H mice, which are *H-2^k*, respond to (T,G)-A--L and (H,G)-A--L as do CBA mice (also *H-2^k*), i.e., they respond poorly to (T,G)-A--L. C3H.SW mice, which are congenic with C3H/DiSn mice (similar to C3H mice) and which are *H-2^b*, do not respond to these antigens in the same manner as C3H mice. C3H.SW mice respond well to (T,G)-A--L and poorly

to (H,G)-A--L, as do C57 mice, which are also H-2^b. Similar results were obtained with B10.BR mice, which are congenic with C57Bl/10 ScSn mice, and A.BY mice, which are congenic with A/WySn mice (similar to A/J mice). An examination of Table III suggests that the ability to respond well or poorly to (T,G)-A--L or (H,G)-A--L can be bred in or out of a strain by selecting for H-2^b in the case of (T,G)-A--L and for H-2^a or H-2^k in the case of (H,G)-A--L.

Linkage Studies.—The hypothesis that the ability to respond well to (T,G)-A--L and (H,G,)-A--L is genetically linked to the H-2 locus was tested by correlating H-2 type with antibody response in a segregating population. In

TABLE III

Response of Congenic Strains of Mice to Synthetic Polypeptide Antigens

Strain*	H-2 type	Response	
		(T, G)-A--L	(H, G)-A--L‡
		%	%
A/J	a	10 (6–15)	66 (29–81)
A.BY	b	78 (62–87)	—
C57	b	69 (51–77)	0
C57Bl/10	b	32 (7–62)	—
B10.Br	k	7 (2–14)	28 (7–55)
C3H	k	10 (0–31)	44 (17–78)
C3H.SW	b	79 (52–91)	0

* Five males and five females of each strain.

‡ Responses to (H, G)-A--L were titered with (T, G)-A--L 509-^{125}I, by virtue of the extensive cross-reactions of these antigens, and therefore give lower per cent antigen bound values than with the homologous antigen (2).

the case of (T,G)-A--L, the population tested was a (CBA $\times$ C57) $F_1 \times$ CBA backcross. In this subline, approximately half of the animals respond well to (T,G)-A--L and half of them respond poorly (1). In addition, since the cross is that of an H-$2^b/H$-2^k heterozygote by an H-$2^k/H$-2^k homozygote, one-half of the offspring would be expected to be H-$2^b/H$-2^k heterozygotes, and one-half would be H-$2^k/H$-2^k homozygotes. The results of these tests are shown in Table IV. 14 out of the 15 mice which responded to (T,G)-A--L were H-2^b-positive, while 22 out of 23 nonresponders were H-2^b-negative, i.e., were H-$2^k/H$-2^k homozygotes. This results confirms the initial hypothesis that the ability to respond to (T,G)-A--L is a genetic trait which is linked to the H-2 locus. The finding of two recombinant animals in 38 animals tested could be due either to failure to immunize these animals properly, or to mistakes in H-2

typing. It is not possible to reimmunize the animals and be certain of the results. The *H-2* typing was repeated three times in all mice, with the same results each time. Breeding tests of the recombinant animals are currently in progress, and if their offspring conform to their designated genotype, it will

TABLE IV

*Linkage of (T, G)-A--L Response to H-2^b^**

(CBA × C57)F₁ × CBA	$H-2^b$	
	Positive	Negative
Responders		
8 (21–75%)	8	0
7 (22–74%)	6	1
—	—	—
15	14	1
Nonresponders		
10 (2–12%)	0	10
13 (0%)	1	12
—	—	—
23	1	22

Total recombinants = 2/38.

* The results given are those of two separate experiments.

TABLE V

*Linkage of (H, G)-A--L Response to H-2^k^*

(CBA × C57)F₁ × C57	$H-2^k$	
	Positive	Negative
Responders*		
6 (10–45%)	6	0
Nonresponders		
14 (0%)	1	13

Total recombinants = 1/20.

* Responses to (H, G)-A--L were titered with (T, G)-A--L 509-I^125^. (2)

indicate that there is a definite low recombination rate between the two traits. Such a result would exclude the possibility that *H-2* antigens affect the immune response to (T,G)-A--L and (H,G)-A--L. Results of similar testing for linkage of ability to respond to (H,G)-A--L with the *H-2^k^* marker are shown in Table V. Of six animals that gave a detectable response to (H,G)-A--L, all six were

H-2^k/H-2^b heterozygotes. Of 14 animals which gave a low or undetectable response to (H,G)-A--L, 13 were H-2^b/H-2^b homozygotes and one was an H-2^k/H-2^b heterozygote. This result again confirms the initial hypothesis and indicates that ability to respond to (H,G)-A--L is linked to the H-2 locus.

DISCUSSION

The finding that the ability to respond to (T,G)-A--L, in either a primary or secondary response, can be transferred by transferring responder spleen cells into irradiated nonresponder recipients indicates that the major genetic difference between responder and nonresponder strains of mice is expressed in one or more types of cells in a spleen cell population, and in all likelihood is directly related to the process of antibody formation. Nothing can be said concerning the type of cell responsible, since it is probable that the heterogenous spleen cell population used contained large lymphocytes, small lymphocytes, plasma cells, macrophages, and quite possibly primitive stem cells. Experiments utilizing purified cell populations to transfer the antibody response to synthetic polypeptide antigens are in progress. Initial experiments will utilize purified macrophages and purified peripheral blood lymphocytes.

The demonstration that the major genetic factor controlling ability to respond to synthetic polypeptide antigens is linked to the H-2 locus and can be localized to the IXth mouse linkage group (7, 8), is unexpected. Since there appears to be an approximate 5% recombination rate between the two genetic markers, it seems unlikely that H-2 antigens themselves are directly involved in the genetic control of the ability to respond to synthetic polypeptides. The IXth mouse linkage group includes the H-2 locus (7), the gene controlling the thymus-leukemia antigen (8), and the gene controlling *Serum Substance* (8). It is possible that genes controlling other cell surface antigens, perhaps on lymphocytes or macrophages, will be detected and placed in this linkage group. Such surface antigens might affect the cell's ability to interact with (T,G)-A--L and (H,G)-A--L. It has already been pointed out (1) that it is unlikely that the genetic control of the ability to respond to (T,G)-A--L is due to sharing of antigenic determinants between (T,G)-A--L and the nonresponder animal's self antigens. Were this the case, nonresponsiveness would be dominant in the F_1, since the F_1 would possess all the nonresponder self antigens (1). Further evidence on this point comes from two sources. First, we have tested two C57/Bl6 anti-CBA isoantisera, one C57/Bl anti-C3H and one C3H.SW anti-C3H isoantiserum all made against spleen cells, and these do not bind (T,G)-A--L. Second, we have recently found (unpublished data) that C57 fetal liver cells can transfer the ability to respond well to (T,G)-A--L into irradiated CBA recipients. Were cross-reaction with CBA self antigens a factor in the poor response of CBA's to (T,G)-A--L, such a result would not be possible, since C57 fetal liver cells would be tolerant of CBA antigens.

This result also rules out the unlikely possibility that the genetic control *is* due to "cross-tolerance," but responsiveness is dominant in the F_1 because the responsible CBA self antigen is recessive in the F_1.

It is also possible that the observed genetic effects are *not* mediated by cell surface antigens, but by other mechanisms, such as the ability of macrophages to "process" the antigen, or the presence in the genome of structural genes coding for a particular antibody combining site complementary to (T,G)-A--L. The recent report that susceptibility to some types of murine leukemia in some strains of mice is a genetic trait which is also linked to the *H-2* locus (9) raises the possibility that susceptibility or resistance is mediated by ability to synthesize neutralizing antibody, and the further possibility that the IXth linkage group includes a large chromosome region which is in some way related to antibody formation.

SUMMARY

The transfer of spleen cells from (C3H $\times$ C57Bl/6) F_1 mice, capable of responding to (T,G)-A--L, into irradiated C3H parental recipients, normally incapable of responding to (T,G)-A--L, transfers the ability to make either a primary or secondary immune response to this synthetic polypeptide antigen. This localizes the genetic control of the ability to respond to the spleen cell population and indicates that the genetic control is exerted upon a process directly related to antibody formation. Studies with congenic strains of mice and linkage studies in segregating backcross populations show that the ability to respond to (T,G)-A--L and (H,G)-A--L is linked to the *H-2* locus and can thus be localized to the IXth mouse linkage group.

Note Added in Proof: Of the three possible recombinant animals noted in Tables IV and V, two were infertile. The third animal was *not* a recombinant, since progeny testing and reimmunization showed that this animal was an $H\text{-}2^b/H\text{-}2^k$ heterozygote capable of responding well to (T,G)-A--L.

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