GENETIC CONTROL OF THE IMMUNE RESPONSE

A SELECTIVE DEFECT IN IMMUNOLOGIC (IgG) MEMORY IN NONRESPONDER MICE*

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The antibody response of inbred strains of mice to immunization with many synthetic branched multichain polypeptides is determined by an autosomal, codominant gene, *immune response-1* (*Ir-1*) (1).[1] This gene locus lies within the murine major histocompatibility locus, *H-2* (2). For example, $H\text{-}2^{b/b}$ strains respond well to poly-L(Tyr,Glu)-poly-D,L-Ala--poly-L-Lys [(T,G)-A--L] and poorly to poly-L(His, Glu)-poly-D,L-Ala--poly-L-Lys [(H,G)-A--L]; $H\text{-}2^{k/k}$ strains respond poorly to (T,G)-A--L and well to (H,G)-A--L, and F_1 ($H\text{-}2^{k/b}$) hybrids respond well to both antigens. When the polypeptide (T,G)-A--L is administered in complete Freund's adjuvant (CFA) for the initial immunization, both $H\text{-}2^{b/b}$ ("responder") and $H\text{-}2^{k/k}$ ("nonresponder") strains slowly develop anti-(T,G)-A--L antibodies over the next several weeks, with the $H\text{-}2^{b/b}$ strain antibody levels slightly greater than those of the $H\text{-}2^{k/k}$ strain. A more striking difference occurs, after a second antigen challenge with (T,G)-A--L in buffered saline, when antibody titers of $H\text{-}2^{b/b}$ mice rise rapidly while those of the $H\text{-}2^{k/k}$ mice remain constant or even decline slightly. If the polypeptide is first electrostatically complexed to a charged protein carrier (such as methylated bovine serum albumin [MBSA]) and then used in the CFA regimen described above, both strains produce a high antibody response to the polypeptide after a secondary challenge with the insoluble carrier-polypeptide complex in aqueous suspension. Analysis of the kinetics of antibody formation in this system may be complicated by the effects of adjuvant on lymphoid (3)

[1] *Abbreviations used in this paper:* ABSA, acetylated bovine serum albumin; CFA, complete Freund's adjuvant; DNP, dinitrophenyl; (H,G)-A--L, poly-L(His, Glu)-poly-D,L-Ala--poly-L-Lys; *Ir-1, immune response-1*; MBSA, methylated bovine serum albumin; 2-Me, 2-mercaptoethanol; MeR, 2-mercaptoethanol-resistant; MeS, 2-mercaptoethanol-sensitive; PBS, phosphate-buffered saline; PLL, poly-L-lysine; (T,G)-A--L, poly-L(Tyr,Glu)-poly-D,L-Ala--poly-L-Lys.

tissue, and by the possibility of persistent release of low doses of antigen from a CFA depot. In order to eliminate any effects of adjuvant, mice were immunized and subsequently boosted with (T,G)-A--L in saline only. The resulting antibody titers were determined as a function of time after antigen challenge for primary, secondary, and tertiary responses. The pattern of immune response to this regimen has revealed differences between responders and nonresponders that were not evident from previous studies. Although both responder and nonresponder strains produced equally good IgM anti-(T,G)-A--L antibodies, only the responder strains were able to produce 7S IgG antibody to that antigen. A model accounting for the observed results on the basis of expression of the *Ir-1* gene in a thymus-derived cell population is presented, and subjected to test in the accompanying paper.

Materials and Methods

Young adult mice of both sexes were obtained from the following sources: C3H/HeJ from the Jackson Laboratory, Bar Harbor, Maine; C3H/HeTex from Texas Inbred Mice Company, Houston, Texas; and C3H/HeJ and C3H/DiSn (origially obtained from Dr. G. D. Snell) from our own breeder colony at Stanford. C3H.SW mice, a strain congenic with the C3H/DiSn mice and differing only at the *H-2* locus, were also obtained from our own colony. (C3H mice are H-$2^{k/k}$ and are (T,G)-A--L nonresponders, while C3H.SW mice are H-$2^{b/b}$ and are (T,G)-A--L responders.)

(T,G)-A--L 52 (mol wt 180,000, kindly provided by Dr. Michael Sela, Department of Chemical Immunology, Weizmann Institute of Science, Rehovot, Israel) was prepared in phosphate-buffered saline (PBS) to yield concentrations of from 0.001 to 100 μg/0.06 cc volume. Mice were immunized and given booster doses of antigen intraperitoneally with 0.06 cc of the appropriate antigen concentration. In all cases, the same dose of antigen was given as a secondary or tertiary stimulus as had been given in the initial immunization. Animals were bled from the retro-orbital sinus or the tail at the times indicated in the text. Equal aliquots of heparinized plasma from each animal within each experimental group were pooled and kept frozen until used for titration of antibody.

Antibodies were assayed by a modification of techniques previously described (4). (T,G)-A--L 52 and (T,G)-A--L 509 (mol wt 232,000, also provided by Dr. M. Sela) are completely cross-reacting antigens from different preparative batches and are interchangeable in this assay system (H.O. McDevitt and F. C. Grumet, unpublished observations). 50 μl of antigen solution containing 0.01 (or 0.005) μg (T,G)-A--L 509 labeled with ^{125}I was incubated at 37°C for 1 hr with 25 μl of a 1:10 (or 1:25) dilution of an antisera pool. 50 μl of the appropriate dilution of a polyvalent rabbit-anti-mouse gamma globulin was then added and the mixture incubated for 2 hr at 37°C, and then centrifuged for 15 min at 10,000 g at 4°C. 50 μl of supernatant was counted in a well-type gamma scintillation counter and results were expressed as per cent antigen bound in the precipitated complexes. Sera from nonimmunized mice or mice given only PBS were used as controls to define a mean null antigen-binding level and gave zero $\pm7.0\%$ (±2 sd) antigen binding.

Disulfide bond reduction of antisera with 2-mercaptoethanol (2-Me) was carried out by incubating plasma pools with equal volumes of 0.1 M 2-Me for 30 min at 37°C, and then diluting the mixture with PBS for immediate titration as described above.

Whole body X-irradiation was performed using a Philips X-ray machine (Philips Electronic Instruments, Mt. Vernon, N.Y.) calibrated at 15 ma and 250 kv to deliver 80 R/min for 10 min.

Sera with ^{125}I-labeled IgG or IgM myeloma markers were chromatographed on 0.6 × 21 cm columns of Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) equilibrated with 0.45 N PBS. Effluent aliquots were collected and tested for optical density at 280 mμ, and counted for radioactivity of the myeloma marker proteins. After concentration by the quantitative addition of dry Sephadex G-25 (fine), aliquots were titered for anti-(T,G)-A--L activity as previously described.
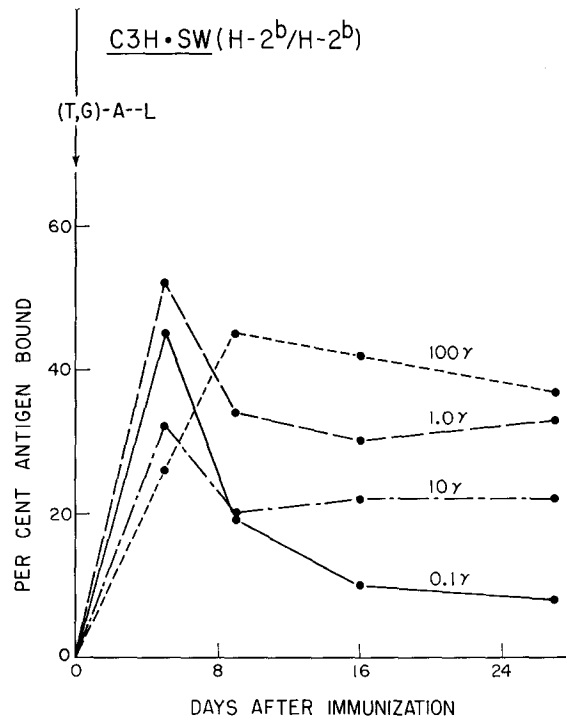
RESULTS

Primary Response.—Figs. 1 *a* and 1 *b* show the primary antibody response of C3H.SW (responder and C3H (nonresponder) mice given graded doses of (T,G)-A--L in PBS on day zero. Except for the highest antigen dose, both responder and nonresponder mice demonstrated similar antibody production with an initial peak during the 1st wk and persistent antibody levels over the next 3 wk. Responder mice receiving 100 μg of (T,G)-A--L developed a moderately delayed response that remained persistently elevated. In both strains the maximum early response occurred with the 1.0 μg dose. Lower antigen doses of 0.01 and 0.001 μg (T,G)-A--L produced responses that were quantitatively lower but otherwise parallel go the 0.1 μg response.
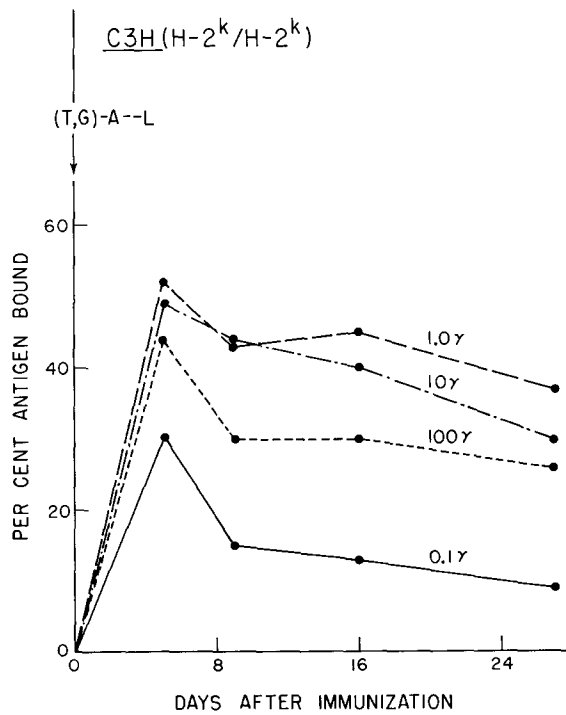
Secondary and Tertiary Responses.—A group of C3H.SW and C3H mice were boosted at 7 and 30 days after their primary immunization, and bled serially for measurement of both total antigen binding and 2-Me-resistant (MeR) antigen-binding capacity. The results of these assays are presented in Figs. 2 *a*–2 *d*. Each figure shows the per cent of antigen bound by untreated and by 2-Me-treated plasma for both responder and nonresponder mice given the same doses of antigen. Both strains of mice produced antibody responses during the 1st wk after immunization that were composed almost entirely of 2-Me-sensitive (MeS) antibody. After the first booster dose (secondary response), neither strain developed another MeS antibody peak and nonresponders also failed to develop any MeR anti-(T,G)-A--L antibody. In contrast, the secondary response in C3H.SW mice shows a rapid rise in MeR antibody that appears to be dose dependent up to a plateau at 10 μg. After a tertiary injection of antigen, a transient fall in plasma antibody in all nonresponders was followed by a weak MeR antibody response that was inversely related to antigen dose (i.e., only the 0.1 μg (T,G)-A--L-immunized mice showed a significant amount of MeR antibody). Once again, there was no prominent MeS antibody peak in either strain. Responder C3H.SW mice showed a slightly more vigorous dose-related MeR antibody response to the tertiary stimulus than was seen in their secondary responses. Additional mice of each strain given 0.01 and 0.001 μg (T,G)-A--L on days zero, 7, and 32 failed to show any response to the secondary or tertiary antigen injection.

In another experiment, Figs. 3 *a*–3 *d*, mice given a second injection of antigen on day 30 developed secondary responses similar to those after the 7th day secondary responses previously described. Low dose immunization (0.01 and 0.001 μg) followed by a secondary stimulus at day 32 again failed to induce any

A

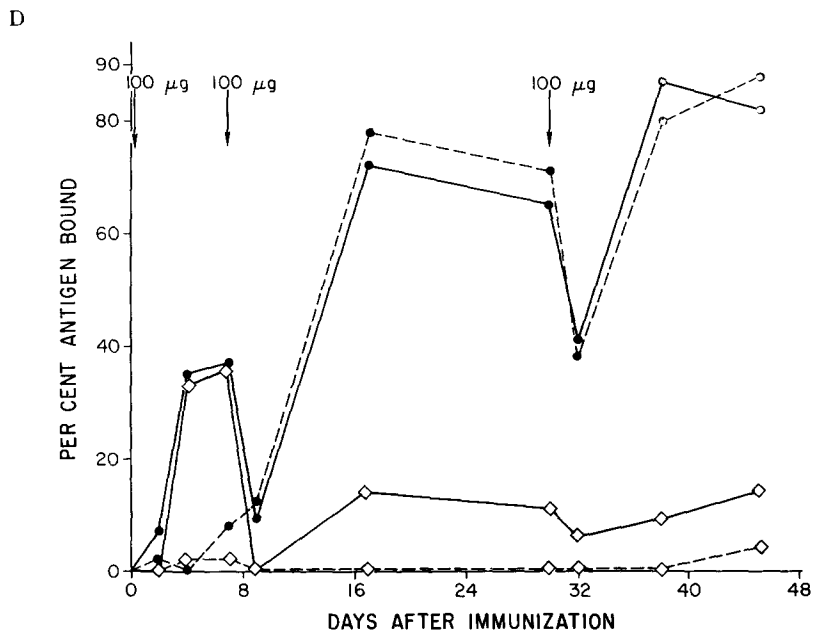C3H·SW(H-2^b/H-2^b)

(T,G)-A--L

PER CENT ANTIGEN BOUND

60

40

100 γ

1.0 γ

10 γ

20

0.1 γ

0

0   8   16   24

DAYS AFTER IMMUNIZATION

B

C3H(H-2^k/H-2^k)

(T,G)-A--L

PER CENT ANTIGEN BOUND

60

40

1.0 γ

10 γ

100 γ

20

0.1 γ
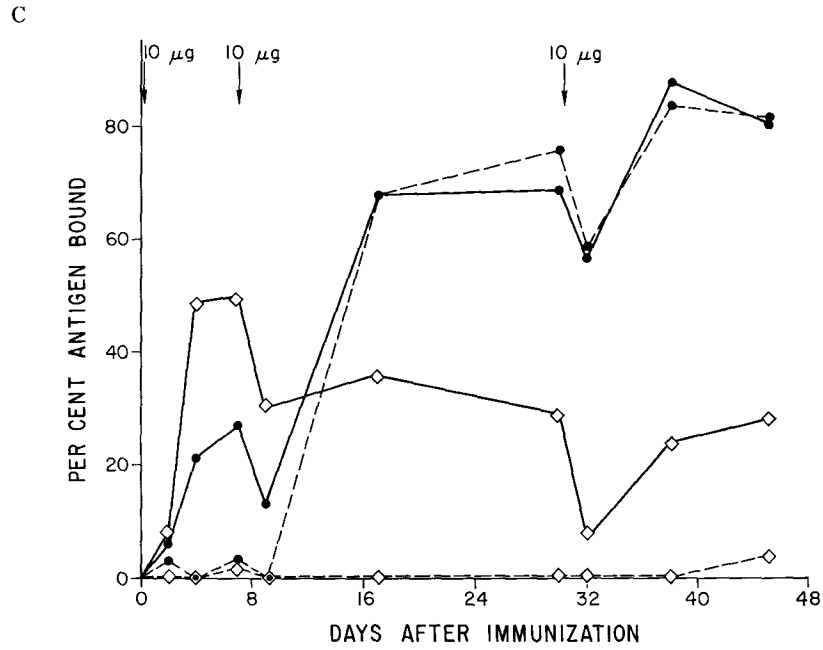
0

0   8   16   24

DAYS AFTER IMMUNIZATION

FIGS. 1 a and 1 b. Antibody response of (a) C3H.SW responder or (b) C3H nonresponder mice to a single challenge at day zero of (T,G)-A--L over a dose range of 0.1–100 μg of antigen. Each point represents a plasma pool of five mice.

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A

B
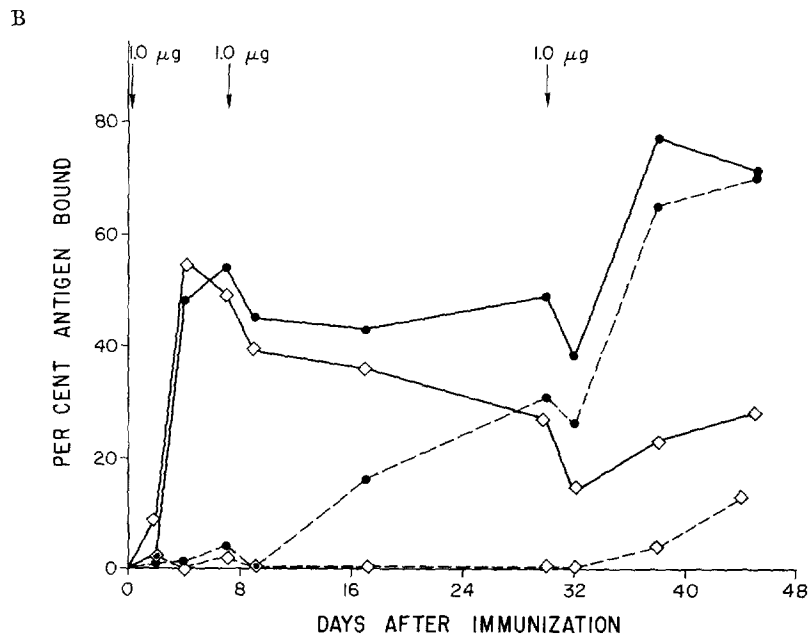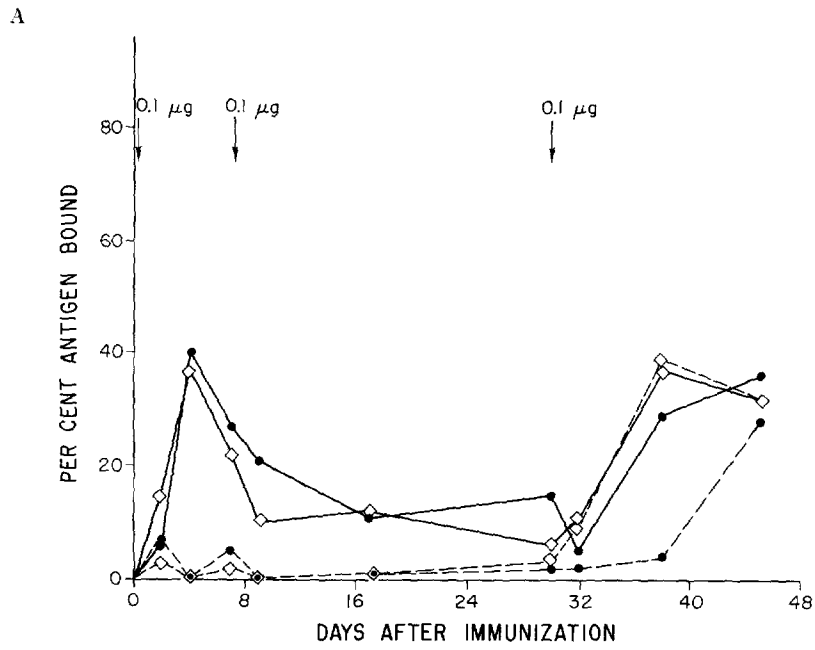
Figs. 2 *a* and 2 *b*.   Total (——) and MeR (-----) antibody response of C3H.SW responder (-◇-) and C3H nonresponder (-●-) mice to primary (day zero), secondary (day 7), and tertiary (day 30) challenge with (T,G)-A--L at (*a*) 0.1 μg and (*b*) 1.0 μg doses of antigen. Each point represents a plasma pool of five mice.

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C

10 μg   10 μg   10 μg

80

PER CENT ANTIGEN BOUND

60

40

20

0

0   8   16   24   32   40   48

DAYS AFTER IMMUNIZATION

D

90

100 μg   100 μg   100 μg

80

PER CENT ANTIGEN BOUND

60

40

20

0

0   8   16   24   32   40   48
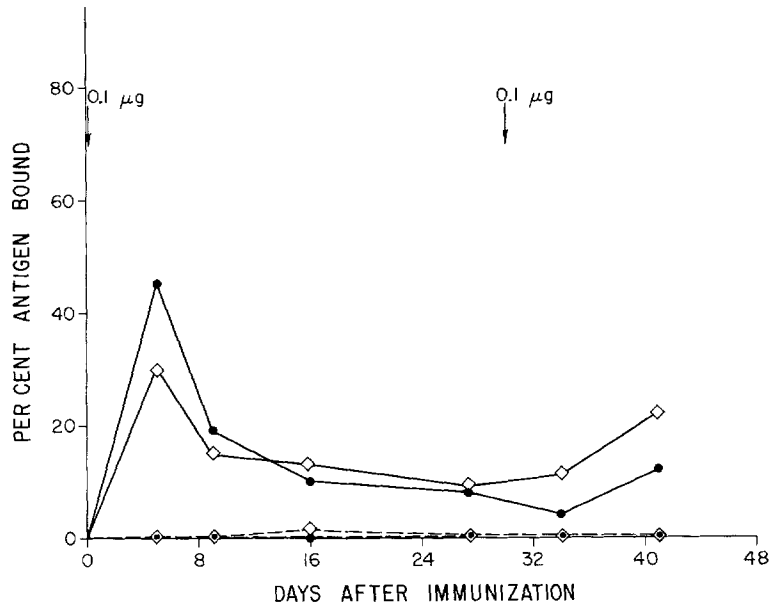
DAYS AFTER IMMUNIZATION

Figs. 2 c and 2 d. Total (——) and MeR (-----) antibody response of C3H.SW responder (-◇-) and C3H nonresponder (-●-) mice to primary (day zero), secondary (day 7), and tertiary (day 30) challenge with (T,G)-A--L at (c) 10 μg, and (d) 100 μg doses o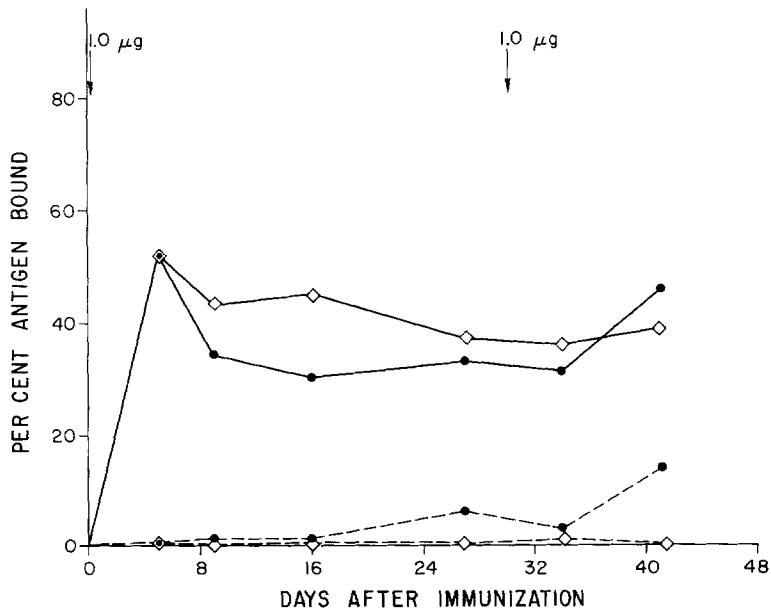f antigen. Each point represents a plasma pool of five mice. Fig. 2 d reprinted by copyright permission from *Ann. N.Y. Acad. Sci.* 1971. **190**.

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A

B

Figs. 3 a and 3 b.   Total (———) and MeR (-----) antibody response of C3H.SW responder
(-◇-) and C3H nonresponder (-●-) mice to primary (day zero) and secondary (day 30)
challenge with (T,G)-A--L at (a) 0.1 μg and (b) 1.0 μg doses of antigen. Each point represents a
plasma pool of five mice.

116

C

10 μg

10 μg

80

PER CENT ANTIGEN BOUND

60

40

20

0

0   8   16   24   32   40   48

DAYS AFTER IMMUNIZATION

D

100 μg

100 μg

80

PER CENT ANTIGEN BOUND

60

40

20
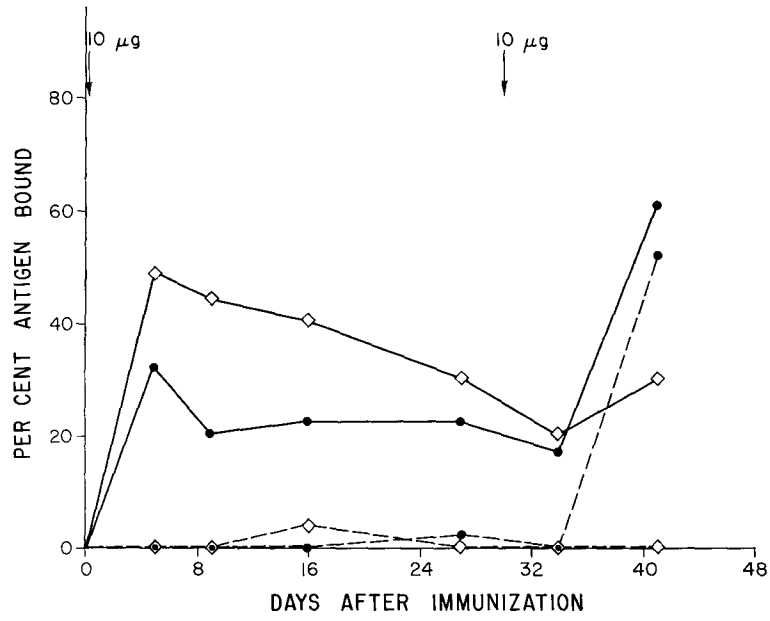
0

0   8   16   24   32   40   48
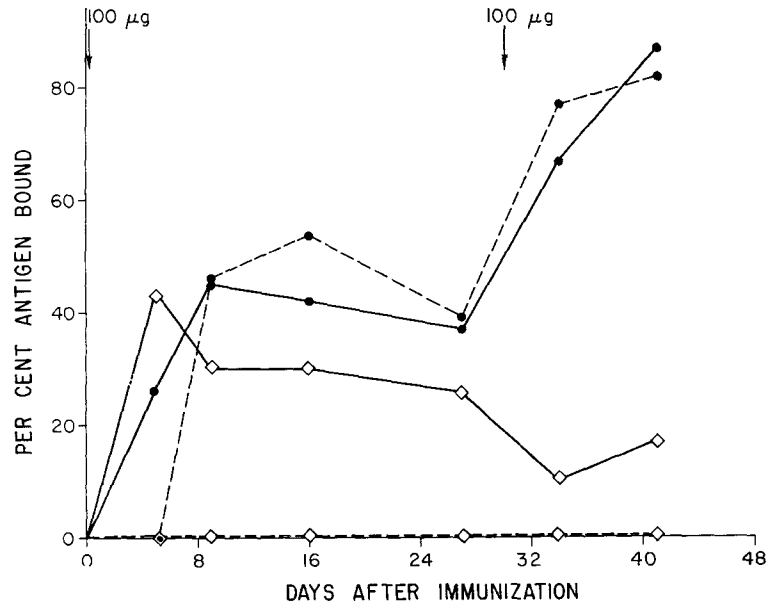
DAYS AFTER IMMUNIZATION

Figs. 3 c and 3 d. Total (———) and MeR (-----) antibody response of C3H.SW responder (-◇-) and C3H nonresponder (-●-) mice to primary (day zero) and secondary (day 30) challenge with (T,G)-A--L at (c) 10 μg and (d) 100 μg doses of antigen. Each point represents a plasma pool of five mice.

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detectable secondary response in either strain. It should be noted that only responder mice receiving 100 μg (T,G)-A--L developed MeR antibodies during the 2nd wk after immunization, before any further injection of antigen was given.

Characteristics of the MeS Antibody Response.—

Radiosensitivity of the MeS response: Four C3H.SW and four C3H mice were mmunized intraperitoneally with 1.0 μg of (T,G)-A--L in PBS 4 hr after re-
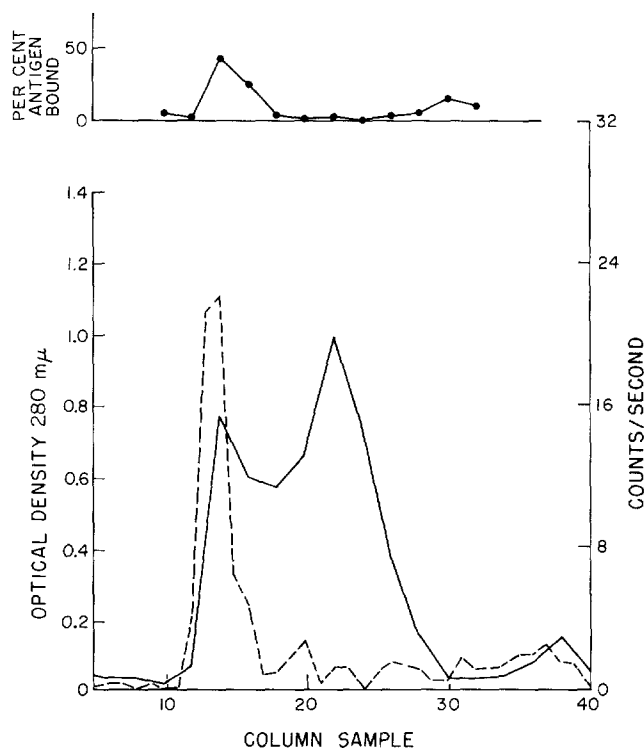

Fig. 4 *a*.   Sephadex G-200 column chromatography of MeS anti-(T,G)-A--L antisera from C3H nonresponder mice bled 3 days after immunization. For each effluent aliquot, the optical density at 280 mμ (——) and the per cent antigen bound (-●-) were compared to the radioactivity (----) of an IgM ^{125}I-labeled myeloma marker.

ceiving 800 R whole body X-irradiation. None of these irradiated animals had detectable antibody to (T,G)-A--L when tested 6 days later.

Column chromatography: Fig. 4 demonstrates the results of column chromatography of anti-(T,G)-A--L antisera on Sephadex G-200. The anti-(T,G)-A--L antisera applied to the column consisted of a pool of plasma from four C3H mice immunized with 10 μg of (T,G)-A--L and bled 3 days later. This pool at a 1:25 dilution gave 25% antigen binding for total antibody activity and 3% antigen binding after 2-Me treatment. Only the untreated antiserum was

applied to the column. The ^{125}I-labeled myeloma markers (kindly provided by Dr. Leonard Herzenberg, Department of Genetics, Stanford University) were GPC8, a γG_{2a} myeloma, and MOPC-104C, a γM myeloma protein. From the figure it is clear that all of the antibody activity of this completely MeS antiserum pool chromatographed with the IgM and not with the IgG marker.

Antibody inhibition: Anti-(T,G)-A--L antisera and normal mouse sera were
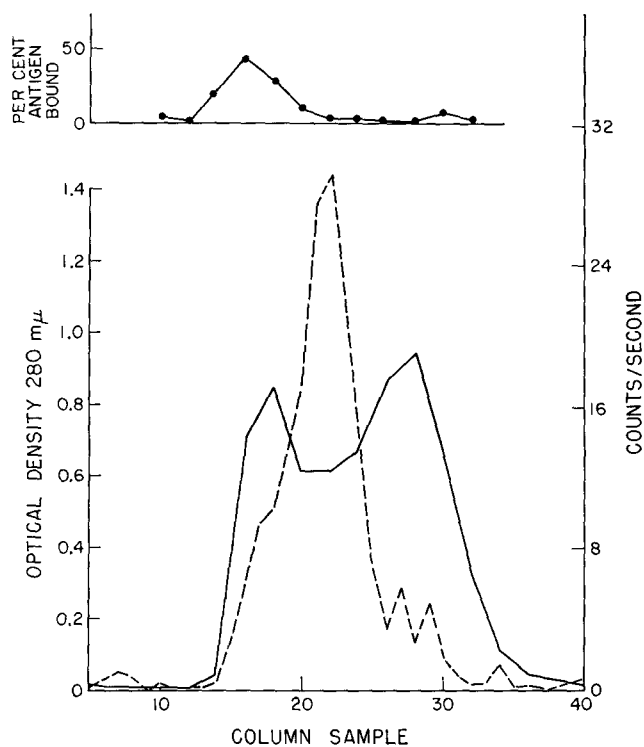

FIG. 4 b.   Sephadex G-200 column chromatography of MeS anti-(T,G)-A--L antisera from C3H nonresponder mice bled 3 days after immunization. For each effluent aliquot, the optical density at 280 mμ (——) and the per cent antigen bound (-●-) were compared to the radioactivity (-----) of an IgG ^{125}I-labeled myeloma marker.

incubated with equal volumes of undiluted goat–anti-mouse IgM antisera[2] (also kindly provided by Dr. Leonard Herzenberg) for 1 hr at 37°C and then overnight at 4°C. The mixture was then centrifuged for 15 min at 10,000 g at 4°C, and the supernatant removed for titration in the usual manner. Only the MeS antibody was inhibited by the anti-IgM antiserum as shown in Table I.

The MeS antibodies have thus been shown to belong to the IgM class of

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[2] This serum produced only a single γM line in immunelectrophoresis against normal mouse serum.

immunoglobulins on the basis of column chromotography and reactivity with specific antisera. Antibodies that were MeR will be considered to be primarily of the IgG class. The demonstration that the MeS antibody response is radiation sensitive is support for the belief that this is a true primary immune response.

TABLE I

Anti-IgM Antiserum Inhibition of (T, G)-A--L Binding by MeR and MeS Anti-(T, G)-A--L Antisera

| Anti-(T, G)-A--L serum | % Antigen binding | | % Antigen binding after inhibition with anti-IgM |
	Total	MeR	
C3H.SW	1	0	0
C3H	1	0	1
C3H.SW	33	0 ·	4
C3H	36	0	1
C3H.SW	57	55	69

DISCUSSION

The kinetics of antibody formation in the immune response to (T,G)-A--L was studied by immunizing responder and nonresponder inbred strains of mice with antigen in aqueous solution. Four significant results were observed. First, both strains demonstrated equally good primary responses consisting of rapid formation of IgM antibody during the 1st wk after immunization. Except for the highest antigen dose in responder mice (which elicited IgG antibody before a secondary stimulus), all animals maintained detectable IgM anti-(T,G)-A--L antibodies without IgG anti-(T,G)-A--L antibody formation for an additional 3 wk. The duration of the IgM response is considerably longer than the half-life (0.5 days) for that class of immunoglobulin, implying continued production of IgM anti-(T,G)-A--L antibodies for a period of at least 1 month after immunization (5).

Second, the most striking difference between responder and nonresponder mice was seen after secondary or tertiary antigen challenge: responders rapidly developed high titers of IgG antibody, while nonresponders produced almost none. At the higher doses of antigen ($\geq$ 10 μg), this difference was qualitative, with nonresponders failing to mount even the feeble IgG response they showed after lower dose ($\leq$ 0.1 μg) tertiary antigen challenge.

Third, not only were nonresponder mice incapable of producing appreciable IgG antibody, but they also failed to produce a significant increase in their IgM antibody after secondary or tertiary antigen challenge.

Fourth, when challenged with high doses of antigen (100 μg), responder mice developed IgG antibody without the need for further antigen challenge.

Any hypothesis attempting to define the mechanism of action of the *H-2-* linked *Ir-1* gene must account for these results. Three reasonable alternative

mechanisms may be postulated. The first would localize the gene defect in non-responders to the marrow-derived precursors of antibody-producing cells, the so called B-cells (6). The most significant evidence against this hypothesis is the ability of nonresponder mice immunized with (T,G)-A--L complexed to MBSA in CFA, to produce IgG anti-(T,G)-A--L antibody in titers equal to that of responder mice (immunized with (T,G)-A--L or with MBSA-(T,G)-A--L in CFA) (7). Although there are no definitive studies to prove that MBSA-(T,G)-A--L-induced antibodies have specificity identical to that of (T,G)-A--L-induced antibodies, data available from serial dilution titrations suggest similarity between these two antibody populations (8). Further evidence on this point comes from a parallel system in guinea pigs, where a histocompatibility-linked gene controlling the ability to respond to poly-L-lysine (PLL) or to haptens on that molecule has been demonstrated (9). Complexing dinitrophenyl (DNP)-PLL to a carrier protein, acetylated BSA (ABSA), induces high titer anti-DNP-PLL antibodies in the nonresponder strain 13 animal (10). Responder guinea pigs immunized with DNP-PLL and nonresponder guinea pigs immunized with DNP-PLL-ABSA produce high titers of anti-DNP-PLL antibodies that are of equal affinity as measured by fluorescence-quenching techniques. These results support the concept that the nonresponder animals do not lack, in their antibody-producing cells, the ability to synthesize anti-(T,G)-A--L antibody, and that these cells are not the site of the *Ir-1* gene effect.

The second possible mechanism of gene action would focus attention on the early phase of the immune response, i.e., antigen recognition. In this hypothesis, nonresponder mice are unable to recognize (T,G)-A--L as foreign, or in some way are unable to present the antigen (or its "message") to the appropriate antibody-forming cells. Because F_1 (responder X nonresponder) and tetra-parental (responder + nonresponder) mice, who possess all the self-antigens of the nonresponder parent, are good responders it is unlikely that (T,G)-A--L is not recognized as an antigen because of any similarity to a nonresponder "self"--antigen (11). Furthermore, both responder and nonresponder mice mount an equal primary IgM response to the antigen, implying that IgM anti-(T,G)-A--L-producing cells of both types recognize a similar degree of foreignness. Because both strains produce equal IgM primary responses, it is reasonable to assume that antigen processing and/or presentation to IgM antibody-forming cells is the same in each strain. If the *Ir-1* gene effect is to be expressed during antigen presentation, then different or additional processing steps for antigen presentation to IgG antibody-forming cell precursors must be postulated. The gene defect in nonresponder strains could thus be an absence of an enzyme or cell surface component or cell population that is essential to the process of antigen recognition for cells destined to produce IgG antibodies.

The third possible mechanism would localize the *Ir-1* gene defect slightly after antigen recognition in the immune response sequence. In this hypothesis, antigen recognition and presentation to IgM-specific antibody-producing pre-

cursor cells would be the same for both responders and nonresponders. These cells or their progeny could then begin to make an IgM response, and in a responder strain could subsequently switch over to an IgG response. In non-responders, the absence of the enzyme or cell surface component or cell population necessary for inducing the shift (to IgG) in an antigen-stimulated, IgM-producing cell population would limit that animal to an IgM response only (except, of course, when the antibody-forming cell is "tricked" by a carrier protein such as MBSA into switching to IgG antibody formation). Evidence that specific cells may be initially committed to producing IgM antibodies, then enter a brief period of production of both IgM and IgG, and finally stabilize as IgG producers comes from several sources and will not be discussed here (12–15). Data available in the (T,G)-A--L system does not yet permit distinction between separate IgM and IgG precursors as opposed to a single precursor capable of switching from IgM to IgG.

The conversion from IgM to IgG antibody production in responders given a single injection of 100 µg (T,G)-A--L must be considered in any comparison of responses of different classes of immunoglobulins. It is clear that an antigen dose threshold between 10 and 100 µg of (T,G)-A--L had to be exceeded before a single antigenic challenge would induce IgG antibody formation. The 100 µg primary antigen dose thus functioned effectively as a combined primary and secondary immunization. If very early bleeding (less than 1 wk) and low dose (10 µg or less) antigen variables had been omitted, the 100 µg (T,G)-A--L response in C3H.SW could have been interpreted as a pure IgG "primary" immune response. In itself, a pure IgG response would constitute strong evidence against a cellular IgM to IgG antibody shift. It is important, therefore, that any "pure" IgG immune responses be properly analyzed over a wide dose range during the earliest phases of the immune response to preclude the presence of a low-level early IgM response.

One further interesting characteristic of the population of cells producing IgM antibodies is most apparent in nonresponder mice, where secondary antigen challenge fails to induce either an IgG response or a further IgM antibody response. The absent IgM secondary response in responders might at least partly be attributable to the negative feedback exerted by the IgG antibodies produced in that strain (16). This mechanism probably is not effective, however, in the nonresponder strain which showed no IgG antibody production. It is also possible that the initial antigen challenge exhausted or paralyzed the IgM anti-(T,G)-A--L antibody-producing cells in the nonresponder mice. Cell transfer studies currently in progress should help in excluding one of these possibilities.

Because only normal animals were utilized in this study, one must exercise great caution in analyzing results with reference to the current concept of the immune response based on thymus-derived (T) cell interaction with bone

marrow (B) cells (6). Nevertheless, the effect of thymectomy on the immune response to many antigens is a selective depression of IgG but not IgM antibody (17–22). This selective depression bears a striking similarity to the pattern of anti–(T,G)-A--L antibody formation in our nonresponders. Thus, responder mice show a "normal" pattern of IgM followed by IgG antibody formation, while nonresponders appear to be functionally thymectomized with respect to the specific antigen (T,G)-A--L. The results of a detailed study of the effects of thymectomy on the antibody response to (T,G)-A--L (presented in the subsequent report) support this concept (23). Although other mechanisms have not been excluded, the data presented here, in conjunction with previous studies, are consistent with the hypothesis that the *Ir-1* gene effect is exerted through thymus-derived cells which influence the conversion of (antibody-producing, bone marrow–derived) B-cells from IgM to IgG production.

SUMMARY

The kinetics of antibody formation after immunization with the synthetic polypeptide poly-L(Tyr,Glu)-poly-D,L-Ala--poly-L-Lys [(T,G)-A--L] in aqueous solution were studied in genetically high (H-2^b) and low (H-2^k) responder strains of mice. During the 1st wk after immunization both strains developed brisk primary responses consisting of IgM antibody. With subsequent antigen challenge, only the high responder mice showed immunological memory, producing high titers of IgG antibody. In contrast, the low responder mice continued to make a persistent low level of lgM antibody and appeared unreactive to secondary or tertiary antigen challenge. These data are consistent with the hypothesis that the *immune response-1* gene [controlling response to (T,G)-A--L] exerts its effect on the immune response at the time of switchover from IgM to IgG antibody production.

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