ANTIBODY RESPONSE OF INBRED MOUSE STRAINS TO ORDERED TETRAPEPTIDES OF TYROSINE AND GLUTAMIC ACID ATTACHED TO MULTICHAIN POLYALANINE OR POLYPROLINE

TYR-TYR-GLU-GLU IS A MAJOR DETERMINANT OF THE RANDOM POLY-(TYR, GLU)-POLY$_{DL}$ALA--POLYLYS*

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The antibody response to a great number of immunogenic systems has been found to be under genetic regulation (1–3). Previous studies on the genetic control of immune responsiveness to multichain synthetic polypeptides were performed with antigens derived from multichain poly-DL-alanine and multichain poly-L-proline to which short sequences of glutamic acid together with tyrosine, histidine or phenylalanine were attached (4–9). These short peptides were obtained by random polymerization of *N*-carboxyamino acid anhydrides (10), and therefore they possess different sequence combinations. Studies with such polypeptides do not enable us to determine whether low responder mice produce antibodies to fewer determinants than the high responders, whether they elicit less antibody to all the determinants, or whether there are differences in the affinity of the antibodies formed (1–9). It was, therefore, desirable to prepare a series of ordered peptides which would represent most of the possible combinations in the random polypeptides.

In the present report, we compare the immune response potential of different mouse strains to ordered tetrapeptides of tyrosine and glutamic acid, attached to multichain poly-DL-alanine and multichain poly-L-proline. Tyrosine and glutamic acid were chosen for the ordered peptides since the random polypeptide poly(LTyr, LGlu)-poly$_{DL}$Ala--poly$_{L}$Lys, designated (T, G)-A--L,[1] is a common immunogen in the study of the genetic control of the immune response (1–4). The results of this study indicate that Tyr-Tyr-Glu-Glu is an important determinant in the random polypeptide (T, G)-A--L, whereas a

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[1] *Abbreviations used in this paper:* A--L, multichain poly-DL-alanyl--poly-L-lysine; G-T-T-G, glutamyl-tyrosyl-tyrosyl-glutamic acid; Pro--L, multichain poly-L-prolyl--poly-L-Lysine; (T, G)-A--L, poly(LTyr,LGlu)-poly$_{DL}$Ala--poly$_{L}$Lys; (T, G)-Pro--L, poly(LTyr,LGlu)-poly$_{L}$Pro--poly$_{L}$Lys; T-G-T-G, tyrosyl-glutamyl-tyrosyl-glutamic acid; T-T-G-G, tyrosyl-tyrosyl-glutamyl-glutamic acid.

different pattern of immune response is obtained when two other tetrapeptides of tyrosine and glutamic acid are tested. It is also shown that Tyr-Tyr-Glu-Glu is essentially immunologically silent in (Tyr-Tyr-Glu-Glu)-Pro--L, similar to the lack of expression of the random (T,G) in (T,G)-Pro--L.

Materials and Methods

Immunogens.—The immunogens used in this study contained the following ordered tetrapeptides of L-tyrosine and L-glutamic acid: tyrosyl-tyrosyl-glutamyl-glutamic acid, abbreviated T-T-G-G; tyrosyl-glutamyl-tyrosyl-glutamic acid, T-G-T-G, and glutamyl-tyrosyl-tyrosyl-glutamic acid, G-T-T-G. The peptides were synthesized using a procedure analogous to that of Ramachandran and Berger (11). The tetrapeptides were prepared from two dipeptides, one of which was blocked at the α-amino-terminus with the tert-butyloxycarbonyl group and activated at the α-carboxyl terminus in the form of *N*-hydroxysuccinimide ester. The second peptide was free at both ends. The dipeptides were synthesized using the same procedure (11), from the appropriate amino acids.

All the peptides were attached to either multichain poly-DL-alanyl--poly L-lysine (A--L) or multichain poly-L-prolyl--poly-L-lysine (Pro--L). The coupling was performed by using the *N*-hydroxysuccinimide ester of the blocked tetrapeptides (12). After removal of the blocking groups (33% HBr in glacial acetic acid), the physicochemical properties of the polypeptides were determined (Table I). Poly(LTyr,LGlu)-polyDLAla--polyLLys, designated (T,G)-A--L, and poly(LTyr,LGlu)-polyLPro--polyLLys, denoted (T,G)-Pro--L, were synthesized and characterized as reported previously (13, 14).

Animals.—AKR/Cu, C3H/HeJ, C3H·SW, C57BL/6, and SJL/J inbred mouse strains were obtained from the Experimental Animal Unit of The Weizmann Institute of Science.

Immunization Procedure.—Mice of the different strains were immunized with each of the polypeptides listed in Table I, by injection of 10 μg of the immunogen in 0.06 ml of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) into the hind footpads. A month later the mice were given a booster injection with 10 μg of the antigen in aqueous solution. The mice were bled 10 days after the booster injection.

Passive Microhemagglutination.—Sheep erythrocytes were formalinized, tanned, and coated with the appropriate antigens (15). The microhemagglutination test (16) was performed on disposable microtiter plates (Cooke Engineering Co., Alexandria, Va.) by twofold serial dilutions of antisera in phosphate-buffered saline (0.15 N NaCl, 0.01 M phosphate buffer, pH 7) containing 0.1% of normal rabbit serum. The plates were incubated at 20°C and read at 2 h and overnight. Only titers at a dilution higher than 1:4 were considered positive, since controls of sera from immunized mice exhibited titers up to 1:4.

RESULTS

Five inbred mouse strains, which represent high and low responders to (T,G)-A--L, were immunized with the three ordered tetrapeptides of tyrosine and glutamic acid attached to either multichain polyproline or multichain poly-DL-alanine (Table I). The antibody responses, expressed as average $\log_2$ of hemagglutination titer of 12 animals from each strain are given in Table II.

As can be seen, the pattern of response of the different inbred mouse strains to the ordered (T-T-G-G)-A--L is similar to that obtained previously to the random polypeptide (T,G)-A--L (1). Thus, C3H·SW and C57BL/6 mice which are high responders to (T,G)-A--L, responded well to T-T-G-G on A--L, whereas C3H/HeJ, AKR/Cu and SJL/J mice, the low responders to (T,G)-

A--L, were found to be low responders to the ordered (T-T-G-G)-A--L as well. Furthermore, the antibodies produced against (T-T-G-G)-A--L cross-reacted well with either (T,G)-A--L or (T,G)-Pro--L, suggesting that T-T-G-G is an important determinant of the random peptides of tyrosine and glutamic acid. It is noteworthy that, as observed with (T,G)-Pro--L (7), upon immunization with (T-T-G-G)-Pro--L the antibodies elicited were specific mainly to the polyproline region of the immunogen, whereas no significant antibody activity was found against the T-T-G-G determinant (Table II).

The immune response potential to either T-G-T-G or G-T-T-G, attached to

TABLE I

Physicochemical Properties of Various Sequential Polypeptides

Polypeptide	Sedimentation coefficient $\times 10^{13}$	Amino acid residues				
		Lys	Ala	Pro	Tyr	Glu
(T-T-G-G)-A--L	4.968	1	22	—	2	2.3
(T-T-G-G)-Pro--L	2.107	1	—	30	2.4	2.4
(T-G-T-G)-A--L	4.742	1	24	—	1.9	2.1
(T-G-T-G)-Pro--L	2.1	1	—	31	1.9	2
(G-T-T-G)-A--L	4.626	1	23	—	2.4	2.4
(G-T-T-G)-Pro--L	2.131	1	—	33	1.9	2

TABLE II

Immune Response of Inbred Mouse Strains to Synthetic Peptides of Known Sequence

Immunizing antigen	Test antigen	Average $\log_2$ of hemagglutination titer				
		C3H·SW	C57BL/6	AKR/Cu	C3H/HeJ	SJL/J
(T-T-G-G)-A--L	(T-T-G-G)-A--L	9*	ND	2	2	2
(T-T-G-G)-A--L	(T-T-G-G-)-Pro--L	8	9	2	2	1
(T-T-G-G)-A--L	(T,G)-A--L	5.6	5.7	2	2	ND
(T-T-G-G)-A--L	(T,G)-Pro--L	5	4.5	ND	ND	ND
(T-T-G-G)-Pro--L	(T-T-G-G)-Pro--L	3	3	4	4	6
(T-T-G-G)-Pro--L	(T-T-G-G)-A--L	2	2	2	2	2
(T-T-G-G)-Pro--L	(T,G)-Pro--L	3	3	4	4	5
(T-T-G-G)-Pro--L	(T,G)-A--L	<2	<2	<2	<2	<2
(T-G-T-G)-A--L	(T-G-T-G)-A--L	6.2	5	7	2	2
(T-G-T-G)-A--L	(T-G-T-G)-Pro--L	5.7	3.6	4.7	3	2
(T-G-T-G)-A--L	(T,G)-A--L	<2	<2	<2	<2	<2
(T-G-T-G)-Pro--L	(T-G-T-G)-Pro--L	5.5	3.4	4.5	4	4.5
(T-G-T-G)-Pro--L	(T-G-T-G)-A--L	3	2	4	2.3	2
(G-T-T-G)-A--L	(G-T-T-G)-A--L	3.2	3.2	3.6	2.4	2
(G-T-T-G)-A--L	(G-T-T-G)-Pro--L	2	3.6	3.6	2.5	3
(G-T-T-G)-Pro--L	(G-T-T-G)-Pro--L	3	4	5	3	4.6
(G-T-T-G)-Pro--L	(G-T-T-G)-A--L	3	2.2	4	2.6	4
(G-T-T-G)-Pro--L	(T,G)-A--L	<2	<2	<2	<2	<2

* Average hemagglutination titers of 12 mice tested.

the multichain polypeptides, was different from that observed after immunization with the random (T,G)-A--L or (T,G)-Pro--L. As can be seen in Table II, AKR/Cu mice which are low responders to (T,G)-A--L, responded well to (T-G-T-G)-A--L and (T-G-T-G)-Pro--L. Moreover, SJL/J mice which were found to be low responders to all the determinants tested previously conjugated with either A--L or Pro--L (1, 3), responded to G-T-T-G when immunized with (G-T-T-G)-Pro--L. No cross-reaction was detected between the antibodies provoked against either T-G-T-G or G-T-T-G and (T,G)-A--L (Table II), suggesting that these determinants are of minor importance or do not exist at all in the random polypeptides.

Table III demonstrates the specificity of the antibodies provoked against the random polypeptide (T,G)-A--L in C3H·SW high responder mice. As can be seen, anti-(T,G)-A--L antibodies cross-react very well with the random (T,G)-Pro--L as well as with the ordered peptide (T-T-G-G) attached to multichain polyproline, suggesting that the majority of the antibodies elicited upon immunization with (T,G)-A--L are specific for the T-T-G-G determinant. In contrast, anti-(T,G)-A--L antibodies cross-reacted very poorly with the ordered (T-G-T-G)-Pro--L immunogen and did not cross-react at all with (G-T-T-G)-Pro--L (Table III), confirming the above mentioned conclusion that these two determinants are of minor importance in the random (T,G)-A--L.

DISCUSSION

One of the important problems in understanding the genetic regulation of the immune response potential to random synthetic polypeptides is to establish the major determinant(s) of all possible amino acid combinations which are responsible for the phenotypic expression of the immune response to the whole immunogen.

In order to elucidate this problem we have immunized mice with three possible tetrapeptide determinants composed of tyrosine and glutamic acid of known sequences. Only one of the three determinants tested, namely, T-T-G-G, was found to have similar properties to those of the random peptide (T,G),

TABLE III

Specificity of Anti-(T,G)-A--L Antibodies

Test antigen	Log_2 of hemmaglutination titer*
(T,G)-A--L	8
(T,G)-Pro--L	7
(T-T-G-G)-Pro--L	7
(T-G-T-G)-Pro--L	3
(G-T-T-G)-Pro--L	2

Log_2 of hemagglutination titers of sera pooled from 10 C3H·SW mice immunized with (T,G)-A--L.

which were expressed both in the pattern of response of the different mouse strains (1) and in the cross-reactivity of antibodies elicited to T-T-G-G with the random (T,G)-A--L (Table II). Furthermore, antibodies elicited upon immunization with the random polypeptide (T,G)-A--L cross-reacted very well with the ordered immunogen (T-T-G-G)-Pro--L (Table III). The finding that no significant antibodies specific to T-T-G-G were detected upon immunization with (T-T-G-G)-Pro--L confirms the similarity of this ordered peptide to the random (T,G). It has been reported that immunization with the random (T,G)-Pro--L provoked antipolyproline antibodies exclusively (7). Indeed, immunization with (T-T-G-G)-Pro--L led to production of antibodies specific for Pro--L (Table II). These results suggest that T-T-G-G is a sequence of major importance in the random peptide (T,G).

The gene controlling the ability to respond to the random polypeptide (T,G)-A--L was found to be linked to the major histocompatibility (*H-2*) locus of the mouse (17). Thus, mice possessing the $H\text{-}2^b$ allele are high responders to (T,G)-A--L, whereas those carrying the $H\text{-}2^k$ antigenic specificity are low responders to the immunogen (17, 1). In addition, SJL mice ($H\text{-}2^s$) are low responders to all the determinants attached to A--L (1, 18). In the present study we have demonstrated that, inded, C3H·SW and C57BL/6 mice ($H\text{-}2^b$) are high responders to (T-T-G-G)-A--L, whereas C3H/HeJ, AKR/Cu ($H\text{-}2^k$), and SJL/J ($H\text{-}2^s$) mice are low responders to this ordered polypeptide. A genetic study using the F_1 hybrids between the high and low responder strains and the backcrosses of F_1 mice and the parental strains is now under progress to establish the linkage to *H-2*.

It should be noted that the $\log_2$ of hemagglutination titers elicited by the low responder mice after immunization with (T-T-G-G)-A--L never exceeded 2. Since this titer is considered to be negative (see Materials and Methods) it appears that mice of the $H\text{-}2^k$ type are nonresponders to (T-T-G-G)-A--L rather than low responders as was observed after immunization with (T,G)-A--L. However, we hope to confirm these results using a more sensitive assay.

It is not yet known whether the low hemagglutination titers found in C3H/HeJ and AKR/Cu mice to (T-T-G-G)-A--L are due to lack of antibody production or to antibodies with low affinity. While no affinity measurements could be done on antibodies elicited to the random (T,G)-A--L due to the possible heterogeneity of the antigenic determinants, the knowledge that T-T-G-G is an important determinant in this immunogen permits the performance of affinity studies.

The two additional tetrapeptides tested, T-G-T-G and G-T-T-G, appear to play a minor role as determinants in the random peptide (T,G), as could be deducted from the different pattern of response observed after immunization with these peptides attached to the multichain carriers and from the observation that antibodies to these ordered peptides did not cross-react with (T,G)-A--L. Moreover, anti-(T,G)-A--L antibodies cross-reacted very poorly with the

ordered determinant T-G-T-G when attached to Pro--L, whereas they did not cross-react at all with (G-T-T-G)-Pro--L (Table III).

The fact that immunization with T-G-T-G or G-T-T-G, conjugated with Pro--L, leads to antibodies specific for the determinants (Table II) confirms the suggestion that these determinants are of minor importance in the random (T,G), since no antibodies specific to the determinants are elicited following immunization with either the random (T,G)-Pro--L or the ordered (T-T-G-G)-Pro--L (Table II) (7).

It is noteworthy that SJL mice produce antibodies specific for the determinant when immunized with (G-T-T-G)-Pro--L. This mouse strain did not elicit upon immunization antibodies specific to any of the random peptide determinants tested when attached to polyproline (7, 9). These mice did not respond to (G-T-T-G)-A--L, as expected from their inability to react with A--L as a carrier (1, 3, 19).

SUMMARY

Five inbred mouse strains which represent high and low responders to the random synthetic polypeptide poly(LTyr,LGlu)-polyDLAla--polyLLys, designated (T,G)-A--L, to which the immune response is controlled by an *H-2*-linked gene, were immunized with three ordered tetrapeptides composed of tyrosine and glutamic acid attached either to multichain poly-DL-alanine or to polyproline. Only one of the three antigenic determinants, namely tyrosyl-tyrosyl-glytamyl-glutamic acid (T-T-G-G), resembled the random peptide (T,G) in the pattern of immune responses elicited against it, and in the cross-reactivity of the specific antibodies with (T,G)-A--L. The immune response pattern to the other two ordered tetrapeptides, T-G-T-G and G-T-T-G, was different from that obtained with (T,G)-A--L, and no cross-reactivity was detected between the antibodies provoked with these peptides and (T,G)-A--L. Thus, it is suggested that T-T-G-G is a major determinant in the random (T,G)-A--L.

REFERENCES

1. McDevitt, H. O., and B. Benacerraf, 1969. Genetic control of specific immune responses. *Adv. Immunol.* **11**:31.
2. Benacerraf, B., and H. O. McDevitt. 1972. The histocompatibility-linked immune response genes. *Science* (*Wash. D. C.*). **175**:273.
3. Mozes, E., and G. M. Shearer. 1972. Genetic control of immune responses. *Curr. Top. Microbiol. Immunol.* **59**:167.
4. McDevitt, H. O., and M. Sela. 1966. Genetic control of the antibody response. I. Demonstration of determinant-specific differences in response to synthetic polypeptide antigens in two strains of inbred mice. *J. Exp. Med.* **122**:517.
5. McDevitt, H. O., and M. Sela. 1967. Genetic control of the antibody response. II. Further analysis of the specificity of determinant-specific control and genetic analysis of the response to (H,G)-A--L in CBA and C57 mice. *J. Exp. Med.* **126**:969.

6. McDevitt, H. O., and A. Chinitz. 1969. Genetic control of the antibody response and histocompatibility (H-2) type. *Science (Wash. D. C.).* **163**;1207.
7. Mozes, E., H. O. McDevitt, J.-C. Jaton, and M. Sela. 1969. The nature of the antigenic determinant in a genetic control of the antibody response. *J. Exp. Med.* **130**:493.
8. Mozes, E., H. O. McDevitt, J.-C. Jaton, and M. Sela. 1969. The genetic control of antibody specificity. *J. Exp. Med.* **130**:1263.
9. Mozes, E., S. Shaltiel and M. Sela. 1973. Genetic control of the immune response to (H,G)-Pro--L. *Isr. J. Med. Sci.* **8**:650
10. Sela, M. 1966. Immunological studies with synthetic polypeptides. *Adv. Immunol.* **5**:29.
11. Ramachandran, J., and A. Berger. 1971. Synthesis and physicochemical properties in aqueous solution of the sequential polypeptide poly(Tyr-Ala-Glu). *Biopolymers.* **10**:1829.
12. Schechter, B., I. Schechter, J. Ramachandran, A. Conway-Jacobs, M. Sela, E. Benjamini, and M. Shimizu. 1971. Synthetic antigens with sequential and conformation-dependent determinants containing the same L-tyrosyl-L-alanyl-L-glutamyl sequence. *Eur. J. Biochem.* **20**:309.
13. Sela, M., S. Fuchs, and R. Arnon. 1962. Studies on the chemical basis of the antigenicity of proteins. 5. Synthesis, characterization and immunogenicity of some multichain and linear polypeptides containing tyrosine. *Biochem. J.* **85**:223.
14. Jaton, J.-C., and M. Sela. 1968. Role of optical configuration in the immunogenicity and specificity of synthetic antigens derived from multichain polyproline. *J. Biol. Chem.* **243**:5616.
15. Herbert, N. J. 1967. *In* Handbook of Experimental Immunology. D. M. Weir, editor. Blackwell Scientific Publications Ltd., Oxford. 720.
16. Kabat, E. A. 1968. *In* Structural Concepts in Immunology and Immunochemistry. Holt, Rinehart and Winston, Inc., New York. 32.
17. McDevitt, H. O., and M. L. Tyan. 1968. Genetic control of the antibody response in inbred mice. Transfer of response by spleen cells and linkage to the major histocompatibility (*H-2*) locus. *J. Exp. Med.* **128**:1.
18. Mozes, E., E. Maron, R. Arnon, and M. Sela. 1971. Strain-dependent differences in the specificity of antibody responses toward lysozyme. *J. Immunol.* **106**:862.
19. Lichtenberg, L., E. Mozes, G. M. Shearer, and M. Sela. 1974. The role of thymus cells in the immune response to poly(Tyr,Glu)-polyDLAla-polyLys as a function of the genetic constitution of the mouse strain. *Eur. J. Immunol.* In press.