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Immune Responses of Inbred Guinea Pigs Against Random Terpolymers Containing L Glutamic Acid and L Alanine[1, 2] (39042)

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The studies of Benacerraf *et al.* (1, 2) and Ben Efraim and Maurer (3) have shown that guinea pigs of strain 2, but not strain 13, have the immune response (IR) gene to respond against the random polymer $G^{60}A^{40}$ (GA),[3] whereas guinea pigs of strain 13 have the IR gene required to respond against the random polymer $G^{50}T^{50}$ (GT). Studies with the more complex terpolymer poly(Glu^{60}-$Ala^{30}Tyr^{10}$), GAT^{10}, which contains many determinants composed of the three amino acids, have shown that although both strains of guinea pigs recognized the complete polymer, they reacted against different determinants within the same molecule (4).

The present investigation was undertaken with random polymers containing the α-L amino acids glutamic acid and alanine and changes in concentration or substitution of the α-L tyrosine residues in GAT^{10}, to learn more about the nature of the 'tyrosine' requirements for the responsiveness of strain 13 guinea pigs to the polymers. The polymers employed contained reduced amounts of tyrosine, i.e. GAT^{4}, the substitution for L tyrosine of α-D tyrosine residues, GAT(LLD), or α-L lysine residues, GAL^{10}, and the chemical modification (nitration) of α-L tyrosine residues, ($GAT\text{-}NO_2$).

The studies will show that strain 2 guinea pigs, irrespective of the nature of the third substituent, responded against all of the above polymers which have GA as part of the molecule. In contrast, strain 13 guinea pigs did not respond to any of the polymers referred to above.

Materials and Methods. Polymers. The random polymers containing different amounts of glutamic acid, alanine, tyrosine (L and D) and lysine were prepared by the polymerization of the *N*-carboxy anhydrides of the specific amino acids (2, 5) (Table I). The nitration of the polymer GAT^{10} was performed by the procedure of Sokalovsky *et al.* (6). A complete study of the physicochemical and immunochemical properties of this derivative has been presented (7).

For use in the antigen binding assay (see below) the polymers GA and GAL^{10} were tyrosylated as previously described (8). The polymers containing tyrosine were iodinated with ^{125}I according to the chloramine-T procedure (9). In all iodination reactions carrier iodide was added to the carrier-free $Na^{125}I$ solution in a quantity equivalent to the amount involved in diiodination of 10% of the available tyrosine residues in the polymer. Unreacted ^{125}I was removed by extensive dialysis against distilled water until no ^{125}I was detected in the dialyzing medium.

Specific activity of polymers. The ^{125}I content and specific activity of all iodinated polymers were determined on an aliquot of a solution in which the nitrogen content was previously analyzed by the Markham modification of the Kjeldahl procedure (10). The specific activity of the ^{125}I labeled polymers varied from 0.4 to 0.8 $\mu Ci/\mu g$. At least 90% of the polymer associated ^{125}I was precipitable with homologous rabbit antisera prepared against the noniodinated polymer.

Immunization of animals. Guinea pigs of strains 2 and 13 were either bred in our animal facility from a breeding stock re-

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[3] Nomenclature of polymers is based upon recommendation of IUPAC-IUB Commission on Nomenclature, October, 1967 (Biochem. J. **106**, 577 (1968)). Abbreviations for amino acids used in the text are as follows: tyrosine = T, glutamic acid = G; alanine = A; lysine = L; poly L lysine = PLL. Superscripts refer to mol percent of the amino acid.

TABLE I. POLYMERS EMPLOYED IN PRESENT STUDY.

	Nomenclature employed in text	Average molecular weight
poly(Glu^{60}Ala^{30}Tyr^{10})	GAT^{10}	55,000
poly(Glu^{58}Ala^{38}Tyr^{4})	GAT^{4}	50,000
poly(Glu^{60}Ala^{30}DTyr^{10})	GAT^{10}(LLD)	30,000
poly(Glu^{60}Ala^{30}Tyr^{10})$(NO_2)_{15}$	(GAT^{10}(NO$_2$)	
poly(Glu^{54}Ala^{36}Lys^{10})	GAL^{10}	50,000
poly(Glu^{60}Ala^{40})	GA	35,000
poly(Glu^{50}Tyr^{50})	GT	50,000

ceived from NIH, or obtained from the National Jewish Hospital, Denver, Colorado, or from Dr. M. Chase of Rockefeller University, New York. Outbred Hartley strain guinea pigs were obtained from local breeders. Animals were immunized in the foot pads with 100 μg or 500 μg of polymer in complete Freund's adjuvant containing 0.5 mg per ml Mycobacterium butyricum (Difco Labs). Ten to 14 days later the guinea pigs were bled, and injected intradermally with 100 μg of the polymer in 0.1 ml of solution. After reading of the delayed skin reactions, the animals were boosted intracutaneously with the same amount of polymer in adjuvant. Two weeks later they were bled and skin tested as above. Nonspecific irritation of up to 6 mm was occasionally observed. Erythema and induration greater than 10 mm in diameter was considered a positive skin reaction and the intensity of the reactions was graded as follows: +, 8–15 mm diameter; ++, 15–25 mm diameter; +++, 25 mm diameter. In several instances, as indicated in the Tables, cross skin reactions were performed with related polymers.

Antibody assay. Sera were assayed for antibody activity using a modified Farr-type antigen binding assay employing the ^{125}I labeled polymer (11). A mixture of 0.05 ml of polymer containing 0.001 μg *N* in phosphate buffered saline and 0.025 ml of a 1:5 dilution of the guinea pig sera in 1% bovine serum albumin was placed at 4°. After 60 min, about 0.25 ml of sheep anti-guinea pig globulin was added to precipitate all the guinea pig gamma globulin. The rest of the assay was as described (12). The percent antigen bound was determined by counting the supernatant in a Nuclear Chicago Co. gamma well counter. Appropriate controls of normal guinea pig serum and known guinea pig antisera were always included. Only results of the binding with secondary sera are presented.

Results. Table II presents some representative data obtained with a number of the polymers, indicating that both strains 2 and 13 guinea pigs respond against GAT^{10}. When the concentration of tyrosyl residues in the polymer is reduced to 4 mol%, i.e., GAT^{4}, none of the strain 13 guinea pigs responded even when immunized with 500 μg. However, the strain 2 guinea pigs responded as against GAT^{10} and produced antibody with specificity directed against both GA and GT determinants. The F_1 (2 × 13) guinea pigs and 4/5 Hartley guinea pigs also responded well against the GAT^{4} polymer.

The data obtained with the GAT^{10} (LLD) polymer, where the tyrosine is of D configuration (Table II) and only strain 2 guinea pigs respond, indicated that the recognition of GAT^{10} by the strain 13 guinea pigs is not only associated with the proper *concentration* of GT determinants, but that both amino acids must be of α-L configuration. The additional requirement for free unmodified L tyrosyl residues in GAT^{10} for the strain 13 guinea pigs to respond is also shown by the results obtained with the nitrated GAT^{10} polymer. Although train 13 guinea pigs did not respond, the strain 2 guinea pigs responded against this modified polymer and produced antibody which reacted with both GA and GAT^{10}. Similarly, the substitution of 10 mole % α-L-lysine for tyrosine in the polymer GAT^{10} eliminated the ability of strain 13, but not strain 2 guinea pigs, to react against the polymer. This is related to the facts that the strain 2 guinea pigs have both the Ir-GA and Ir-GL immune response genes, and that the strain 13 guinea pigs have neither of these genes (1, 2).

Discussion. The data presented in this report with random polymers of amino acids emphasize the importance of a sufficient concentration of amino acids constituting the specific recognition determinant (GT), as well as the need for the proper configuration

TABLE II. IMMUNE RESPONSE OF GUINEA PIGS TO Poly(Glu AlA) TERPOLYMERS CONTAINING A THIRD AMINO ACID.

Guinea pig strain	Polymer	Dose injected (μg)	No. animals responding/No. animals tested	Intensity of skin reaction[a]	% Antigen bound[b,c] 2° response
2	GAT^{10}	500	6/6	+++	75 ± 6
					40 ± 5 (GA)
					54 ± 4 (GT)
13		500	5/5	++	50 ± 9
					2 ± 2 (GA)
					30 ± 5 (GT)
2	GAT^{4}	500	3/3	++	46 ± 10
					51 ± 8 (GAT^{10})
					31 ± 11 (GA)
					68 ± 2 (GT)
13		500	0/4	−	6 ± 4
F_1		500	6/6	+++	52 ± 10
					39 ± 9 (GA)
					59 ± 8 (GT)
Hartley		500	4/5	+++	70 ± 5
					38 ± 8 (GA)
					64 ± 14 (GT)
2	GAT^{10} (LLD)	100	3/3	++	75 ± 14
		500	4/4	+++	58 ± 16
13		100	0/3	−	3 ± 3
		500	0/4	−	5 ± 2
2	GAT^{10} (NO^{2})	500	5/5	++	37 ± 14 (GAT^{10})
					48 ± 15 (GA)
13		500	0/10	−	6 ± 3
2	GAL^{10}	100	3/3	+++	42 ± 3
					48 ± 4 (GA)
13		100	0/3	−	4 ± 3
		500	0/4	−	6 ± 2

[a] Delayed skin reactions read 24 hr after injection are graded according to text.

[b] The amount of ^{125}I polymers bound by 1:5 dilution of sera compared to normal sera (See text for assay).

[c] Values obtained with homologous and (cross reacting) polymers as indicated. ( ) Refer to cross reacting polymer.

of the amino acid in the determinants constituting the recognition determinant. The GAT^{10} polymer is immunogenic for both strains 2 and 13 guinea pigs, where recognition of the polymer by strain 13 guinea pigs is via the gene product of the Ir-GT gene, and by strain 2 guinea pigs via the Ir-GA gene product. This need for the appropriate amount of tyrosine is shown by the fact that reduction in the concentration of tyrosine to 4 mole %, the nitration of a considerable number of the tyrosyl residues in the immunogenic GAT^{10}, the replacement of L tyrosine by either D tyrosine or L lysine, eliminated the immunogenicity of these polymers only for the strain 13 guinea pigs but not for the strain 2 guinea pigs which have the Ir-GA gene. When the strain 2 guinea pigs respond to all of the above polymers through the recognition of appropriate GA determinants, they produce a significant concentration of anti GT antibody.

The above studies are analogous to those reported with the responses of strains 2 and 13 guinea pigs to the GLT^{5} and GLT^{15} polymers (13). Strain 2 guinea pigs responded well against both polymers through the recognition of GL determinants in both polymers (Ir-GL gene), but the strain 13

guinea pigs responded only against GLT^{15} polymer and not the GLT^{5} polymer. The explanation offered was that GLT^{5} did not have the necessary concentration of GT determinants required for initiation of the response through interaction with the product of the Ir-GT gene.

Similar restrictions in the responses of strain 2 guinea pigs to random polymers containing different ratios of glutamic acid and alanine have also been obtained in our laboratory. Although strain 2 guinea pigs respond well against $G^{60}A^{40}$, changes in mol ratios of the two α-L amino acids, i.e. $G^{90}A^{10}$, or substitution of either D glutamic acid or D alanine for the L amino acids resulted in nonimmunogenic polymers (Maurer, unpublished observation). This has also been reported on for Hartley guinea pigs (14, 15). Although one refers to immune response genes, such as Ir-GA, Ir-GL, Ir-GT in the random polymers of amino acids, the specific structures of the determinants being recognized are not known and points up the need for further immunogenetic studies with polymers of known sequences of amino acids. Experiments with such sequential polymers are underway in our laboratory.

Summary. The immune responses of the inbred guinea pig strains 2 and 13 have been determined against random terpolymers of L glutamic acid and L alanine and a third amino acid. Strain 2 guinea pigs responded against GAT^{10}, GAT^{10}(LLD), $GAT^{10}(NO_2)_{15}$, GAT^{4}, and GAL^{10}. However, strain 13 guinea pigs responded only against GAT^{10}. The explanation offered is that strain 2 guinea pigs, which have the Ir-GA gene, recognize the polymers via random GA determinants present in sufficient concentration in all of the above polymers. However, strain 13 guinea pigs recognize the GAT^{10} via the Ir-GT gene, and reduction in the concentration of tyrosyl residues below 10 mole % by various procedures alters the concentration of available random GT determinants necessary for interaction with the gene product of the Ir-GT gene.

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1. McDevitt, H. O., and Benacerraf, B., Advan. Immunol. **11,** 31 (1969).
2. Benacerraf, B., and McDevitt, H. O., Science **175,** 273 (1972).
3. Ben Efraim, S., and Maurer, P. H., J. Immunol. **97,** 577 (1966).
4. Bluestein, H. G., Green, I., Maurer, P. H., and Benacerraf, B., J. Exp. Med. **135,** 98 (1972).
5. Katchalski, E., and Sela, M., Advan. Protein Chem. **13,** 243 (1958).
6. Sokalovsky, M., Riordan, J. F., and Vallee, B. L., Biochemistry **5,** 3583 (1966).
7. Odstrchel, G., and Maurer, P. H., Immunochemistry **11,** 15 (1974).
8. Maurer, P. H., and Merryman, C. F., Immunogenetics **1,** 174 (1974).
9. Hunter, W. M., *in* "Handbook of Experimental Immunology" (D. M. Weir, ed.), p. 608, F. A. Davis and Co., Philadelphia, Pennsylvania (1969).
10. Markham, R., Biochem. J. **36,** 790 (1942).
11. Herzenberg, L. A., Warner, N. L., and Herzenberg, L. A., J. Exp. Med. **121,** 415 (1965).
12. Merryman, C. F., and Maurer, P. H., J. Immunol. **108,** 135 (1972).
13. Maurer, P. H., Proc. Soc. Exp. Biol. Med. **147,** 640 (1974).
13. Maurer, P. H., Proc. Soc. Exp. Biol. Med. **147,** 640 (1974).
14. Maurer, P. H., Gerulat, B. F., and Pinchuck, P., J. Exp. Med. **119,** 1005 (1964).
15. Maurer, P. H., J. Exp. Med. **121,** 339 (1965).

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