

ANTIGENICITY OF POLYPEPTIDES (POLY ALPHA AMINO ACIDS)*

XV. STUDIES ON THE IMMUNOGENICITY OF SYNTHETIC POLYPEPTIDES IN MICE

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In recent years there has been much interest in the use of synthetic linear polypeptides as models of proteins (1). These polymers have consisted of 2 or 3 L- α -amino acids of known composition but having random sequence. The observations that these copolymers were antigenic in rabbits (2), guinea pigs (3), and man (4), provided a new tool for the study of the chemical basis of antigenicity of proteins. It was found that the patterns of response obtained varied both between species and within individuals of a species. The present investigations were initiated to extend our knowledge of this variability of the immune response to copolymers in a species where genetic and biochemical studies offered considerable promise.

Materials and Methods

Polymers.—Table I presents the copolymers tested as antigens with their mole per cent composition and average molecular weights. They were prepared, and the molecular weights determined, as described in the references (5, 6). All of the copolymers used were soluble in neutral aqueous media.

Animals.—For most of the studies, random bred albino "Swiss" mice, weighing 18 to 20 gm, and 5 to 6 weeks old, were used. They were obtained from a number of different suppliers: Darwin Laboratories, Brooklyn, (SPF) Carworth Farms, New City, New York, (CF1), and Cam Research, Wayne, New Jersey, (Swiss). Inbred strains of mice were generally obtained from the R. B. Jackson Laboratories, Bar Harbor, Maine; a few were from Darwin Laboratories. They were housed in metal cages, under standard conditions, with food and water *ad lib*, and were kept for at least 1 week after arrival before being immunized.

Immunization.—Based on preliminary experiments the amounts of antigen and schedule of immunization and bleedings used were as follows: a solution of the appropriate polymer

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(10 mg/ml, pH 7.2) was emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Inc., Detroit). Animals were injected with 0.2 ml of the antigen-adjuvant emulsion (1 mg polymer) with a 25 gauge needle. Injections were on the belly or sides, and were given either intradermally or subcutaneously (course I). Two weeks later, the animals were boosted with the same amount of adjuvant (course II). Nine or 10 days later, they were bled 0.25 to 0.5 ml from the retroorbital plexus (II-9 or II-10 bleeding) under light ether anesthesia. The mice were then allowed to recover for 2 to 5 weeks, and boosted (course III) and bled (III-9 or III-10) as for course II.

In a number of cases, as indicated in Results, where there had been no immune response, the animals were reimmunized with another polymer (generally GLA₄₀).¹ Only two courses of immunization were performed as the II-9 bleeding was invariably positive.

TABLE I
Copolymers Studied for Immunogenicity in Mice

Ratio of amino acids in copolymer	Nomenclature of copolymer employed	Lot No.	Average molecular weight
Glu ₉₀ tyr ₁₀	G ₉₀ T ₁₀	M-13	65,000
Glu ₆₀ ala ₄₀	G ₆₀ A ₄₀	M-15	35,000
Glu ₆₀ ala ₃₀ tyr ₁₀	G ₆₀ A ₃₀ T ₁₀	M-18	25,000
Glu ₅₄ ala ₃₆ lys ₁₀	G ₅₄ A ₃₆ L ₁₀	M-47B	50,000
Glu ₆₀ lys ₄₀	G ₆₀ L ₄₀	C-26	70,000
Glu ₆₀ lys ₄₀	G ₆₀ L ₄₀	C-29C	27,000
Glu ₆₀ lys ₄₀	G ₆₀ L ₄₀	C-32	115,000
Glu ₅₈ lys ₃₈ tyr ₄	G ₅₈ L ₃₈ T ₄	M-12	94,000
Glu ₅₈ lys ₃₈ phe ₄	G ₅₈ L ₃₈ Phe ₄	M-16	48,000
Glu ₅₇ lys ₃₈ ala ₅	GLA ₅	M-22	50,000
Glu ₅₄ lys ₃₆ ala ₁₀	GLA ₁₀	M-46	70,000
Glu ₄₈ lys ₃₂ ala ₂₀	GLA ₂₀	M-45	55,000
Glu ₄₂ lys ₂₈ ala ₃₀	GLA ₃₀	M-21	57,000
Glu ₃₆ lys ₂₄ ala ₄₀	GLA ₄₀	M-11	45,000
Glu ₂₄ lys ₁₆ ala ₆₀	GLA ₆₀	M-49	28,000
Glu ₃₆ lys ₂₄ ala ₃₅ tyr ₅	GLAT	M-17E	30,000

The blood was allowed to clot overnight in the cold, spun down at 2000 RPM and the serum collected. All sera were absorbed with an equal volume of packed tanned sheep RBC, for 30 minutes at 37°C, then 2 to 18 hours in the cold. They were then centrifuged and the absorbed sera tested individually, initially by the standard method (7), and later by the Takatsy Micro procedure, employing tanned sheep red blood cells which allowed repeated titration (8). Sera were generally refrigerated, not frozen and were tested within 1 to 2 days following the bleeding. No loss of titer was observed in sera kept refrigerated for up to 1 week, or frozen and thawed.

Testing of Sera.—The method for testing sera, which was found to be the most sensitive and utilized the least amount of serum obtained was the passive hemagglutination of antigen-coated tanned red blood cells (7). Sheep cells in elsevers solution were obtained commercially

¹ In this article, G, L, A, T, and Phe stand, respectively, for the amino acids glutamic acid, lysine, alanine, tyrosine, and phenylalanine. Subscripts refer to mole per cent amino acid in the polymer.

(Probio) and were used within a month. They were washed 3 times with saline- PO_4^2 at 1200 RPM, 0–4°C, and adjusted to 2.5 per cent concentration in PO_4 -saline buffer. An equal volume of freshly prepared 1:20,000 tannic acid was added, and the cells were incubated at 37°C for 15 minutes, with occasional resuspension. The cells were spun down in the cold, washed twice with PO_4 -saline, and appropriate amounts of a 2.5 per cent suspension were spun down in individual tubes and resuspended in 1.0 ml of adjusted saline. One ml of antigen solution containing 1 to 10 mg of polymer was added, and then 4.0 ml of the pH 6.4 PO_4 -saline. The cells were coated at room temperature for 30 minutes, spun down, and washed 3 times with 3.0 ml of 1 to 100 normal rabbit serum (NRS) (previously absorbed with sheep cells and diluted in pH 7.2 PO_4 -saline). They were then resuspended to a concentration of 2.5 per cent.

Parallel titrations with both rabbit and mouse sera indicated that the 2 hemagglutination methods were directly comparable. Appropriate volumes of the sensitized cells were added to serial, two-fold dilutions of the sera to be tested; all dilutions were made in NRS. The lowest dilution of each serum was tested with tanned, uncoated (but washed with NRS) sheep cells, and controls of antigen-coated cells with NRS and known antisera (rabbit or mouse) were also run with each titration. Patterns were read after 3 hours at room temperature and overnight in the cold.

In some cases, it was necessary to use other means than simple tanning of red cells to get the polymers onto the sheep cells. The bis-diazo benzidine (BDB) method, as described by Sehon (9), was used to couple tyrosine-containing polymers to sheep cells. A large batch of BDB was prepared and stored frozen in individual ampules till used. For each antigen, the appropriate ratio of BDB (coupler) to polymer was empirically determined and its efficacy checked, using rabbit antisera known to react with the polymer (2). The appropriate ratio gave satisfactorily reproducible results. Three ml of solution, containing 1 mg polymer, in pH 7.2 saline were mixed with the button of 3 ml of 10 per cent washed cells and 0.5 ml of BDB- PO_4 . After 15 minutes at room temperature, with occasional stirring, the cells were centrifuged in the cold, washed twice with 1 to 100 NRS, and resuspended to 2.5 ml. The agglutination patterns were also read by the method mentioned.

Agar Diffusion.—Where indicated, the Preer double diffusion technique was used as previously described (10). Antigen concentrations of 1 to 20 $\mu\text{g N}$ were used. Tubes were placed in the cold and observed at intervals for 1 month.

RESULTS

The results presented in this paper are almost exclusively based upon the tanned cell hemagglutination method, which has the disadvantage that the polymers which do not contain lysine are not absorbed to the tanned erythrocytes. Most of the polymers used in this study, which contain 10 to 40 mole per cent of lysine are absorbed satisfactorily and agglutination is readily observed. For the others those which contain tyrosine can be coupled with BDB; if the appropriate ratio of cells, coupling agent and polymer is chosen, sufficient antigenic determinant groups are left intact to react with antibody. Cells coupled with $\text{G}_{90}\text{T}_{10}$ or $\text{G}_{60}\text{A}_{30}\text{T}_{10}$, could be agglutinated by rabbit antisera to $\text{G}_{90}\text{T}_{10}$, $\text{G}_{60}\text{A}_{30}\text{T}_{10}$, and $\text{G}_{60}\text{A}_{40}$, as well as mouse antisera to $\text{G}_{60}\text{A}_{30}\text{T}_{10}$.

In the case of antisera to $\text{G}_{60}\text{A}_{40}$, it was necessary to use a cross-reacting pol-

² Saline- PO_4 ; 0.15 M NaCl, 0.15 M PO_4 buffer, pH 7.2. PO_4 -saline; 23.9 ml 0.15 M KH_2PO_4 , 76 ml 0.15 M Na_2HPO_4 , 100 ml saline- PO_4 . NRS; 1 per cent normal rabbit serum, diluted in PO_4 -saline buffer.

ymmer as the homologous polymer could not be adsorbed or coupled to sheep RBC. Therefore, $G_{54}A_{36}L_{10}$ -treated cells or BDB-coupled $G_{60}A_{30}T_{10}$ cells were employed. Rabbit antisera to $G_{60}A_{40}$ readily agglutinated such cells. In addition, passive cutaneous anaphylaxis and precipitin double diffusion in agar (Preer) tests were carried out on the sera from mice immunized with $G_{60}A_{40}$. In all cases, the negative results obtained agreed with the negative HA results. Table II presents the results with Swiss mice obtained from 3 different suppliers. No antibody could be detected in the sera from mice immunized with the copolymers of 2 amino acids. In addition to the standard immunization schedule, other groups of animals were tested for response to $G_{60}L_{40}$ and $G_{60}A_{40}$ following a variety of schedules; e.g., 4 and 12 days after a single injection of

TABLE II
*Response of Swiss Mice to Copolymers**

Copolymer injected	Course II	Course III
$G_{90}T_{10}$	0/20	0/20
$G_{60}A_{40}$	0/38	0/30
$G_{60}L_{40}$		
C-26	0/30	ND
C-29c	0/18	0/16
C-32	0/10	0/10

ND, not done.

* Number responding/number tested.

polymer in adjuvant, several months after 1 or 2 injections, at dose levels of 25 μ g and 10 mg, or polymer in saline solution. None of the mice tested responded. Subsequently, the animals were immunized with GLA_{40} or $GLAT$, and all responded.

The following inbred strains of mice were immunized with $G_{60}A_{40}$ and $G_{60}L_{40}$ (10 to 40 mice per strain): A, $C_{57}Bl/6$, B6AF₁, C_3H/He , BALB/c, 129. There were no responders to the copolymers of 2 amino acids, but a good response was noted in all mice upon reimmunization with GLA_{40} .

Introduction of 4 to 5 mole per cent of a third amino acid into either $G_{60}L_{40}$ or $G_{60}A_{40}$ produced an immunogenic polymer (Table III). Thus, in the former 4 mole per cent tyr or phe ($G_{58}L_{38}T_4$, $G_{58}L_{38}Phe_4$) and in the latter lys or tyr ($G_{54}A_{36}L_{10}$, $G_{60}A_{30}T_{10}$) produced excellent immunogens. The nature of the small amount of the third amino acid introduced made little difference as regards immunogenicity. With the polymers of $(glu_{60}lys_{40})ala_x$, where the per cent ala varied from 5 to 60 mole per cent, only about half of the Swiss-Webster mice responded even after prolonged immunization with GLA_6 . With increasing ala

content in the polymers (GLA₁₀, GLA₂₀), there was an increased percentage of responders (85 to 90 per cent), finally resulting in 100 per cent responders (GLA₃₀, GLA₄₀, and GLA₆₀). The polymer GLAT was also an excellent immunogen, when composed of L-amino acids, but not when the amino acids are of the D configuration (11). The sera from mice immunized with G₆₀A₄₀ and GLA₄₀ were tested by agar diffusion. There was no response to the G₆₀A₄₀, but the GLA₄₀ sera produced single sharp bands. It should be noticed that rabbit sera to these polymers generally gave 1 weak and 1 strong band (2).

TABLE III
Response of Swiss Mice to Polymers of 3 or 4 L- α -Amino Acids

Copolymer	Course II			Course III		
	Responding tested	Responders <i>per cent</i>	Titer*	Responding tested	Responders <i>per cent</i>	Titer*
G ₅₄ A ₃₆ L ₁₀	9/18	50.0	12	11/16	68.8	22
G ₆₀ A ₃₀ T ₁₀	19/21	90.4	28	20/20	100	74
G ₅₈ L ₃₈ T ₄	20/20	100	160	20/20	100	57
G ₅₈ L ₃₈ Phe ₄	20/20	100	67	16/16	100	133
GLA ₅	7/36	19.4	18	17/36	47.1	27
GLA ₁₀	16/27	59.2	14	23/25	92	15
GLA ₂₀	28/33	84.8	52	28/33	84.8	19
GLA ₃₀	8/8	100	36	6/6	100	21
GLA ₄₀	14/14	100	66	10/10	100	161
GLA ₆₀	18/18	100	156	10/10	100	75
GLAT	9/9	100	123	7/7	100	474

* Mean reciprocal hemagglutination titer of responding animals.

DISCUSSION

In considering the results obtained, it is important to consider the specificity and sensitivity of the method employed. Our previous experience (2), as well as the results obtained here with the BDB method indicate that there is considerable specificity for tyrosyl residues in anti-G₆₀A₃₀T₁₀ sera. In the case of BDB coupled polymers, although our results with high titer mouse anti-G₆₀A₃₀T₁₀ indicate that tyr is part of the antigenic determinant (unpublished), there is sufficient tyr (10 mole per cent) in the polymers so that there is no major destruction of determinant groupings. The titers obtained with rabbit antisera by HA paralleled those of PCA or precipitin reactions. Thus, rabbit anti-G₆₀T₁₀ with a PCA titer of 1:5 to 1:10 had an HA titer of 1:4 to 1:8. Furthermore, polymer-coated cells form well defined settling patterns providing clean-cut end-points, even in relatively high serum concentrations. The titers obtained were often fairly low, and sometimes dropped between the second and

third courses of immunization (*i.e.*, $G_{58}L_{38}T_4$ and GLA_{60}). Nevertheless, by PCA or precipitin reactions, high titers were found. Basically, this seems to be due to the fact that under the conditions used for immunization, the response consisted primarily of 7S and not 19S antibody. Generally, the former has relatively poor direct hemagglutinating activity, especially with hyperimmunization, in contrast to the latter (12).

The most interesting finding in this work has been the apparently consistent non-immunogenicity of polymers of 2 amino acids in mice, and the appearance of immunogenicity upon the introduction of a few mole per cent of a third amino acid, without regard to the nature of the third amino acid. It should be noted that Sela's studies with multichain polyalanine provides a picture in agreement with our findings (13). If the side chains consisted of a single amino acid coupled to the non-immunogenic backbone, the polymer was non-immunogenic. However, copolymer side-chains such as glu-his, glu-phe, glu-leu, and glu-lys were immunogenic. In recent studies from our laboratory, introduction of a few moles of a hapten into a copolymer, but not a homopolymer, led to the formation of antihapten antibodies (unpublished).

The pattern of response to these polymers is different in each of the species tested (rabbit, guinea pig, man, mouse). Thus, in the GLA series of polymers in rabbits, GLA_{30} is the best antigen; GLA_{40} is somewhat less effective, and GLA_{60} is distinctly less antigenic. In mice, there seems to be considerable importance of the ala content; *i.e.*, GLA_{40} and GLA_{60} were the best antigens. Quantitative cross-reactions with rabbit and mouse sera to the same polymer indicates that the groupings against which the antibodies are directed are not identical, despite the relative simplicity of the polymers. Our quantitative precipitin studies with mouse antibodies against $G_{60}A_{30}T_{10}$ showed relatively little cross-reaction with $G_{60}A_{40}$ or $G_{54}A_{36}L_{10}$, in contrast to the 80 per cent cross-reaction noted with rabbit anti- $G_{60}A_{30}T_{10}$. Polymers containing tyrosine, such as $G_{90}T_{10}$, gave high precipitin cross-reactions with these mouse sera. A minimal precipitin cross reaction was found with the rabbit anti- $G_{60}A_{30}T_{10}$ with the $G_{90}T_{10}$ antigen. In the case of mouse anti- GLA_{40} , there was no cross-reaction with $G_{60}L_{40}$, and increasing cross-reaction of the GLA polymers with increasing ala content; *i.e.*, GLA_{60} , gave the best cross-reaction. With the rabbit anti- GLA_{40} , $G_{60}L_{40}$ gave 15 per cent cross-reaction, and the best cross-reaction was with GLA_{30} .

SUMMARY

The response of mice to synthetic linear polypeptides of known composition but random sequence has been studied. Neither Swiss mice nor a number of inbred strains could respond to copolymers of only 2 amino acids ($G_{60}L_{40}$, $G_{60}A_{40}$, $G_{90}T_{10}$). Upon introduction of as little as 4 mole per cent of a third amino acid, good immune responses were obtained, regardless of the nature of the

third amino acid. The level of the immune response to a series of glu-lys-ala polymers increased with increasing alanine content of the polymer.

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